

MECHANISM OF ACTION OF CHOLERA TOXIN

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DECLARATION

I certify that,

- (a) I am the author of this thesis, and
- (b) all of the work presented in this thesis was done by me,
unless otherwise indicated in the text.

W. H. J. WARD

PREFACE

The work which is in this thesis was carried out under the supervision of Dr S. van Heyningen at the Department of Biochemistry, University of Edinburgh. Parts of this work have been published. Copies of these papers and abstracts are included at the back of the thesis. My studentship was supported by the Medical Research Council.

I am especially grateful to the following people for their assistance and advice, and for helping to make my time at Edinburgh so enjoyable. Dr Paul Britton, Dr Ian Flynn, Lilian Rankine, Clare Taylor, Avril Thom and Dr Simon van Heyningen. I would also like to thank Val Lyons for typing this thesis.

This work is dedicated to my parents.

ABSTRACT

Cholera toxin binds to the outside of cells and one of its subunits enters to activate adenylate cyclase. In the current work the nature of this action across the membrane was related to the hydrophobicities of cholera toxin and its subunits. Charge-shift electrophoresis showed that the proteins have no hydrophobic surfaces. Amino-acid composition and sequence data suggested that the molecules have no masked hydrophobic regions. Therefore part of cholera toxin may interact with polar molecules during its passage across the membrane.

GTP was required for cholera toxin to catalyze the incorporation of radioactivity from NAD^+ into rat liver membranes. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) followed by autoradiography showed that the principal substrates of the toxin were of $M_r = 42\ 000$ and $47\ 000$. The substrates were digested by proteolytic enzymes and radiolabelling was inhibited by nicotinamide. These results were consistent with reports that cholera toxin transfers ADP-ribose from NAD^+ to polypeptides. After solubilization from rat liver membranes both substrates of cholera toxin occurred in a protein of apparent $M_r \approx 180\ 000$ on gel-permeation chromatography. Rat liver membranes were complemented with detergent extracts from control or cholera toxin-treated membranes. The results of these experiments showed that cholera toxin modified the regulatory component of adenylate cyclase. The action of cholera toxin required NAD^+ and GTP. In all of the experiments, ADP-ribosylation by cholera toxin paralleled activation of adenylate cyclase, suggesting that ADP-ribosylation caused stimulation of the enzyme. Gel-permeation chromatography showed that a tritiated hydrolysis-resistant analogue of GTP (β, γ -imido- $[\delta\text{-}^3\text{H}]\text{guanosine } 5'\text{-triphosphate}$, $[\delta\text{-}^3\text{H}]\text{p(NH)ppG}$) bound to the regulatory component of adenylate cyclase in an essentially irreversible manner. Pretreatment of membranes with saturating concentrations of p(NH)ppG did not inhibit ADP-ribosylation by cholera toxin, showing that the sites of modification were not where guanyl nucleotides were bound. L-arginine methyl ester specifically inhibited the action of cholera toxin, suggesting that the toxin was modifying arginine, or arginine-like, residues. Treatment with phenylglyoxal

inactivated adenylate cyclase. Stimulation of the enzyme by cholera toxin, or by p(NH)ppG, induced increased susceptibility to phenylglyoxal, suggesting that activation produced dissociation of adenylate cyclase.

The substrates of cholera toxin were [^{32}P]ADP-ribosylated and then purified using SDS-PAGE. Each polypeptide was partially digested with proteolytic enzymes, and the radioactive products were analysed by SDS-PAGE followed by autoradiography. In other experiments the cysteine residues on each substrate were blocked and then each polypeptide was exhaustively digested with proteolytic enzymes. The radioactive products were analysed by 'peptide-mapping' followed by autoradiography. These investigations showed that the two substrates of cholera toxin have very similar structures and that each is probably ADP-ribosylated at more than one, but only a few, sites.

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1. INTRODUCTION

1.1 THE PHYSIOLOGY OF CHOLERA

Vibrio cholerae enter the body orally, passing through the stomach to multiply in the small intestine. Cholera is an acute diarrhoea producing dehydration which leads to death. Fluid in which V.cholerae had been cultured was sterilised and then injected into ligated loops of small intestine. Injection of this sterile fluid mimicked the effect of the bacteria in producing a loss of liquid by the intestinal tissue (De, 1959). It was shown that a protein secreted by the bacteria was responsible for inducing the symptoms of the disease (see Field, 1979). This protein is called cholera toxin.

Cholera toxin induces an increase in the level of cAMP in intestinal tissue (Chen et al., 1972). The high cAMP concentrations inhibit absorption of NaCl by villus cells and stimulate active secretion of anions (probably by crypt cells) (Field, 1979). The increase in ionic strength in the lumen of the intestine causes a loss of water by the adjacent tissue resulting in diarrhoea.

Cholera toxin elevates the levels of cAMP by increasing its synthesis rather than by inhibiting its breakdown. The toxin increases basal and hormone-stimulated activities of adenylate cyclase (E.C.4.6.1.1) which is the enzyme that synthesises cAMP (Bennet et al., 1975). Unlike hormones that stimulate the enzyme, cholera toxin has very little tissue specificity activating it in almost all of the eukaryotic systems that have been tested (van Heyningen, 1977a).

Cholera toxin may also affect the enteric nervous system to cause a flow of fluid into the gut (Cassuto et al., 1981).

1.2 THE STRUCTURE OF CHOLERA TOXIN

Cholera toxin is a very stable protein containing neither carbohydrate nor lipid (van Heyningen, 1976 a). The molecule is made up of two types of subunit, A and B, which are not linked covalently. The subunits may be separated by urea, guanidine or detergents (van Heyningen, 1974).

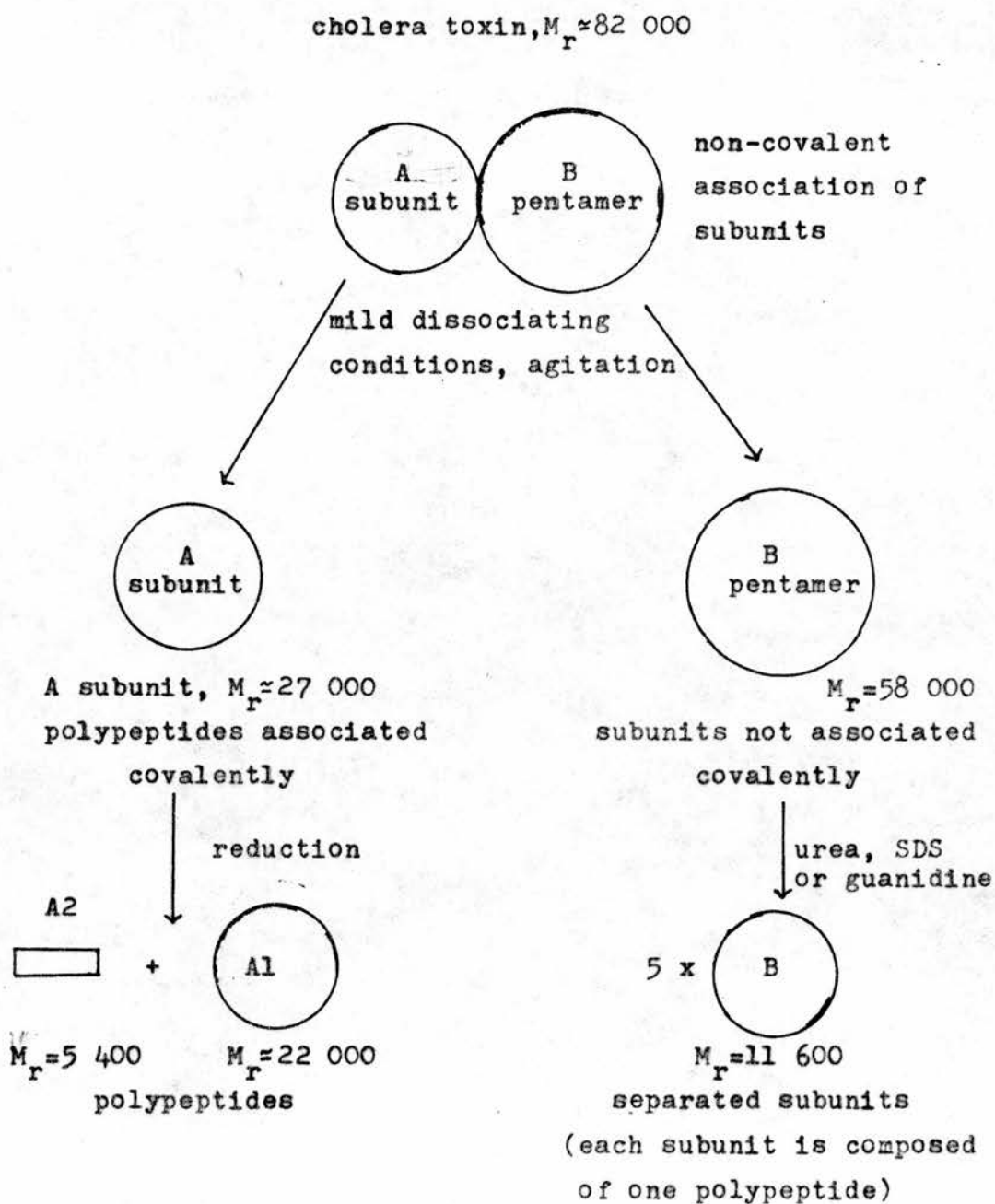
There is one A subunit in each molecule of cholera toxin. The A subunit has a $M_r \approx 27\ 000$ and consists of two polypeptide chains, A1 and A2 ($M_r \approx 22\ 000$ and $5\ 400$ respectively) which are linked by a disulphide bond (van Heyningen, 1977 a; Duffy *et al.*, 1981 a) (fig 1.1). The A subunit is probably synthesised as a single polypeptide chain and then cleaved (Gill & Rappaport, 1979; Duffy *et al.*, 1981 a). The A subunit of cholera toxin, but not the B subunit, activates adenylate cyclase in preparations of broken cells (van Heyningen, 1976 a). Much of the primary structure of the A subunit is known (Lai, 1980; Duffy *et al.*, 1981 b). Purified preparations of the A subunit from cholera toxin stick to glass and to chromatography media (van Heyningen, 1976 a). If the concentration of A subunit is high in these preparations, then the molecule forms aggregations (van Heyningen, 1976 a). These observations suggest that the A subunit is a hydrophobic protein.

Each molecule of cholera toxin contains five B subunits which are linked to each other by strong, non-covalent bonds (van Heyningen, 1976 a). The aggregation of five B subunits is known as choleragenoid. Each B subunit is composed of a single polypeptide chain (van Heyningen, 1976 a). The amino-acid sequence of the B subunits has been elucidated showing that the $M_r = 11\ 600$ (Nakashima *et al.*, 1976). The B subunits interact more strongly with each other than they do with the A subunit. Sodium dodecylsulphate (SDS) causes the A subunit to dissociate from the rest of the toxin, but higher concentrations of SDS are required to separate the B subunits from each other (van Heyningen, 1976 a). The time-course of cross-linking with

Fig. 1.1

The subunit structure of cholera toxin

This figure is explained in section 1.2.



with dimethylsuberimidate showed that the B subunits are closer to each other than they are to the A subunit (Gill, 1976 a). The principles of protein aggregation (see Klotz et al., 1975) allow only one possible three-dimensional arrangement of the subunits of cholera toxin. This is a ring of B subunits with the A subunit above the ring (fig. 1.2). This hypothesis is supported by the results of structural studies of cholera toxin using electron microscopy (Ohtomo et al., 1976) and X-ray crystallography (Sigler et al., 1977).

Treatment of cholera toxin with 2-mercaptoethanol reduces the disulphide bond in the A subunit causing the A1 polypeptide to dissociate from the aggregation of the A2 polypeptide and choleragenoid (Sattler & Weingadt, 1975). Cross-linking studies also showed that the A2 polypeptide links the A1 polypeptide to the complex of B subunits (Gill, 1976 a).

1.3 THE BIOCHEMICAL BASIS OF CHOLERA

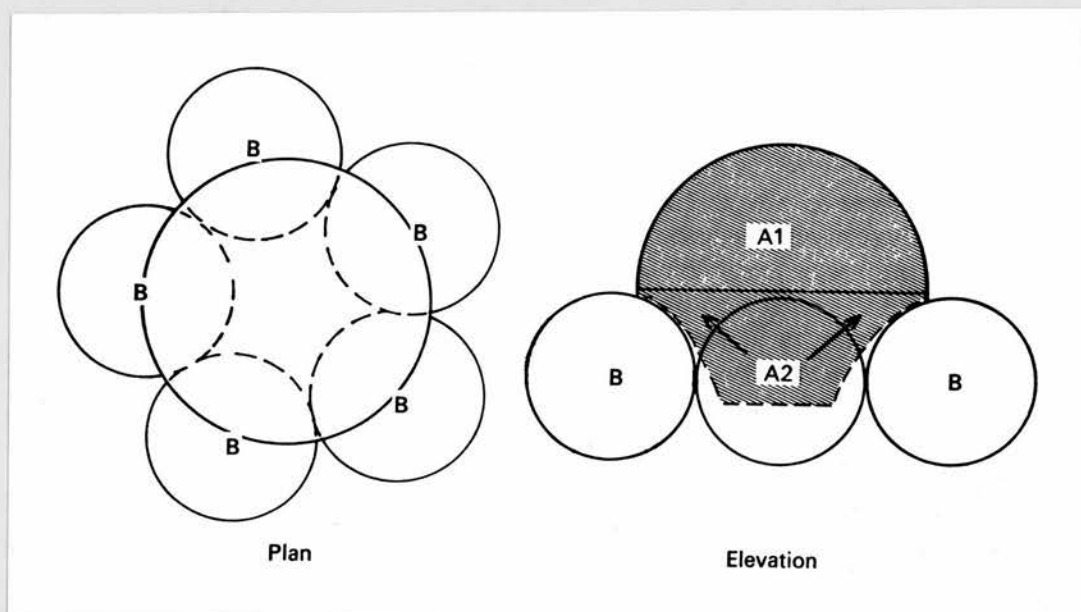
1.3.1 The mechanism by which the active part of cholera toxin enters cells

In cholera, bacteria secrete the toxin into the lumen of the intestine. Cholera toxin then binds rapidly to the outside of cells, but it does not activate adenylate cyclase until at least 30min have passed (van Heyningen, 1977 a). This lag phase is not seen when cholera toxin is incubated with preparations of broken cells (van Heyningen & King, 1975). The A1 polypeptide (or part of it) from cholera toxin activates adenylate cyclase by catalyzing a reaction which occurs at the inner surface of the plasma membrane (see section 1.3.3). The A1 polypeptide must therefore be transported across the cell membrane or, at least, protrude across it. This requirement probably accounts for the lag phase for 30min between the binding of cholera toxin and the activation of adenylate cyclase in intact cells.

Fig. 1.2

The three-dimensional arrangement of protomers in cholera toxin

This figure is taken from van Heyningen (1977 a) and is discussed in section 1.2 of the current work.



1.3.1.1 The binding of cholera toxin to cells

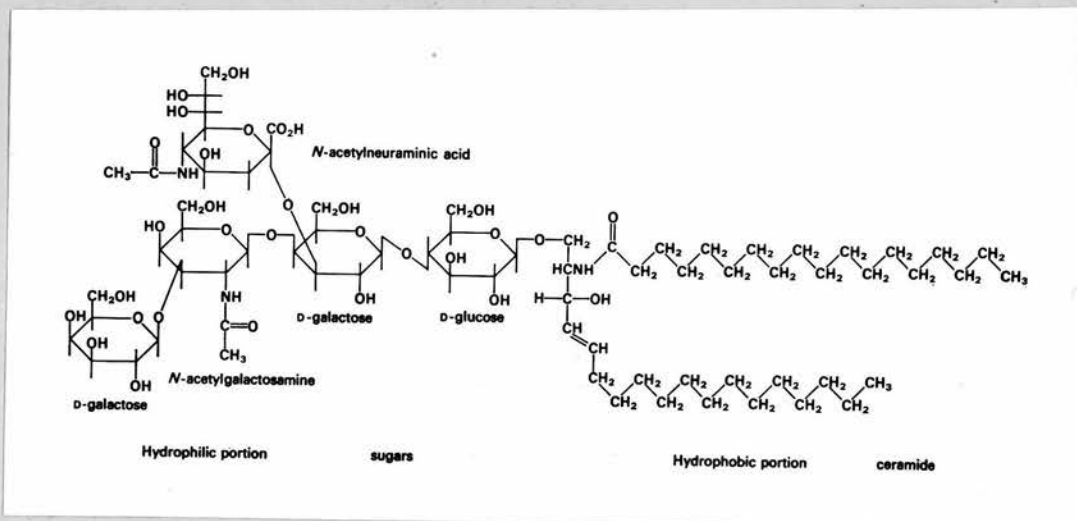
Preincubation of cholera toxin with a mixture of gangliosides decreased the effect of the toxin on intestinal tissue (van Heyningen et al., 1971).

Gangliosides are amphiphilic lipids which are found in all plasma membranes. They have two long hydrophobic chains which dissolve in the membrane, and a hydrophilic carbohydrate part which remains on the outside of the membrane (see van Heyningen, 1977a and fig. 1.3). The B subunit of cholera toxin binds specifically to the oligosaccharide moiety of ganglioside GM1 (van Heyningen, 1977a; Critchley et al., 1979). The carbohydrate portion of ganglioside GM1 may determine the specificity of a larger receptor which is a glycoprotein. Several glycoproteins that bind cholera toxin in vitro have been isolated from the membranes of rat intestinal microvilli (Morita et al., 1980). Pretreatment of cells with a proteolytic enzyme (trypsin) did not decrease their response to cholera toxin (Fishman, 1980a). This suggested that protein which was exposed on the outside of the cells was not required for the function of the receptor. This conclusion was supported by the observation that addition of ganglioside GM1 to cells increased their response to cholera toxin (Fishman, 1980a). Cholera toxin was bound to Balb/c 3T3 cells which were subsequently treated with galactose oxidase then NaB^3H_4 . Incubation with galactose oxidase followed by NaB^3H_4 radiolabels exposed galactose residues. Pretreatment with cholera toxin prevented labelling of galactose residues in ganglioside GM1, but did not protect galactose groups in glycoproteins (Critchley et al., 1982). This showed that, although cholera toxin bound to glycoproteins that had been purified from membranes (Morita et al., 1980), the toxin did not attach to the proteins when they were in the membranes. Ganglioside GM1 remains associated with insoluble material after cells are treated with cholera toxin and then with the detergent Triton X-100. This treatment is thought to solubilize all of the cell except the cytoskeleton (Streuli et al., 1981). Therefore it was suggested that

Fig. 1.3

The structure of ganglioside GM1

This figure is taken from van Heyningen (1977a) and is discussed in section 1.3.1.1 of the current work.



ganglioside GM1 is associated with cytoskeletal proteins (Streuli et al., 1981; Sahyoun et al., 1981a). However preincubation of membranes with cholera toxin causes a great decrease in the amount of ganglioside GM1 which may be extracted using these techniques (Hagmann & Fishman, 1982). This may be because several molecules of ganglioside bind to each molecule of cholera toxin to form insoluble complexes therefore the low extractability of ganglioside GM1 may not be because it is associated with the cytoskeleton as was suggested above.

When purified B subunit or complete cholera toxin bind to ganglioside GM1 changes are observed in the fluorescence and circular dichroism spectra of the proteins (Mullin et al., 1976; Moss et al., 1977a). The oligosaccharide derived from ganglioside GM1 had the same effects as the complete molecule on the proteins (Fishman et al., 1978). This suggested that the binding of ganglioside induced a change in the conformation of the B subunit of cholera toxin. A derivative of cholera toxin was prepared that was labelled with a fluorescent probe in the A subunit but not in the B subunits. This derivative retained the ability of the toxin to activate adenylate cyclase in a preparation of broken cells. Fluorimetric studies showed that the addition of ganglioside GM1 to the modified toxin caused a conformation change in the A subunit (van Heyningen, 1982a).

1.3.1.2 Models for the mechanism of the transmembrane action of cholera toxin

Local perturbation of the membrane by cholera toxin

The binding of cholera toxin to ganglioside GM1 probably aligns the choleraagenoid portion of the toxin parallel to the surface of the cell. The A subunit would be held close to the plasma membrane. Conformation changes (due to the binding of ganglioside) may promote the dissociation of the A subunit from the rest of the toxin. The local increase in

concentration of ganglioside GM1 could perturb the membrane to facilitate the penetration of the A1 polypeptide.

The binding of cholera toxin to ganglioside GM1 does perturb artificial membranes. Cholera toxin induced the release of glucose from liposomes whose membranes contained ganglioside GM1 (Moss et al., 1976a ; 1977b). Bilayers of glycerol mono-oleate (that contained ganglioside GM1) had an increased conductance after treatment with cholera toxin (Tosteson & Tosteson, 1978). Therefore cholera toxin can make membranes permeable to small compounds. The B subunits from cholera toxin have the ability to induce the movement of larger molecules (such as polypeptides) across membranes (see section 1.3.1.3).

Each molecule of cholera toxin can bind several molecules of ganglioside GM1. After cholera toxin has attached to a cell, the toxin-ganglioside complex moves on the surface of the membrane (perhaps until binding becomes multivalent (Fishman & Atikkan, 1980)). A requirement for multivalent binding of ganglioside could explain the lag phase between the attachment of cholera toxin to the plasma membrane and the activation of adenylate cyclase in intact cells. This hypothesis is consistent with the observation that increasing the amount of ganglioside GM1 in the membrane decreased the lag phase (Fishman, 1980b).

The movement of the cholera toxin-ganglioside GM1 complex on the surface of the membrane was dependant upon temperature (Fishman & Atikkan, 1980). Liposomes, whose membranes contained ganglioside GM1, released [^{14}C]glucose after treatment with cholera toxin. The increase in permeability of the membrane was dependant upon time and temperature suggesting that multivalent binding was required (Mullin et al., 1980).

Cholera toxin is a potent activator of adenylate cyclase in HeLa cells. Cholera toxin was bound to HeLa cells by

incubating them together at 4°C. The cells were then treated (at 37°C) with the oligosaccharide from ganglioside GM1. The activation of adenylate cyclase by the toxin was considerably inhibited, and the toxin was displaced from the cells (Fishman & Atikkan, 1980). This implied that multivalent binding of ganglioside is required before cholera toxin can exert its effect across a membrane.

Cells that are deficient in ganglioside GM1 are relatively insensitive to cholera toxin (Fishman et al., 1980). Addition of ganglioside GM1 to such cells increased their susceptibility to toxin. Diacetyl GM1 is a derivative of ganglioside GM1 in which the long hydrophobic chains (fig. 1.3) are replaced by acetyl groups. Under conditions where ganglioside GM1-treated cells and diacetyl GM1-treated cells were binding equal amounts of cholera toxin, the latter cells accumulated more cAMP. The time-lag before the production of cAMP was similar in each set of cells. The two groups of cells had equal affinities for cholera toxin. It was concluded that, although the carbohydrate moiety of ganglioside GM1 bound to cholera toxin, the hydrophobic portion of the glycolipid controlled the interactions between the toxin and the membrane (Fishman et al., 1980).

Experiments using electron spin resonance spectroscopy showed that the photoactive probe [$1-^{14}\text{C}$]/12-(4-azido-2-nitrophenoxy) stearyl glucosamine partitioned into the outer monolayer of the membrane of Newcastle Disease Virus (NDV). On illumination, reactive nitrene residues were produced about 1.3nm from the carboxyl groups on the surface of the membrane (Wisnieski & Bramhall, 1979; Wisnieski et al., 1979). The membrane of NDV is rich in ganglioside GM1. NDV membranes were pretreated with the photoactive probe and then incubated in the dark with cholera toxin. The system was then illuminated. The only part of the cholera toxin that was radiolabelled was the A1 polypeptide. No labelling was detected if the incubation with cholera toxin was at 0°C

(Wisnieski & Bramhall, 1979; Wisnieski et al., 1979). These results confirm the effect of temperature on the action of cholera toxin across membranes. The results of these photoaffinity labelling experiments showed that the B subunits remain at the surface of the membrane, whereas the A1 polypeptide crosses, or extend into, the membrane bilayer. But, as the membrane of NDV is very different to the natural site of action of cholera toxin, it is not known whether this experimental system is representative of the situation in vivo.

Hydrophobic interactions between cholera toxin and the membrane

Holmgren (1980) proposed that the conformation changes that are induced when cholera toxin binds to ganglioside could expose hydrophobic regions of the protein. These areas could then interact with hydrophobic proteins or lipids in the membrane. The entry of the A1 polypeptide into the cell could then be facilitated by one of the following methods.

- (a) The B subunits could unfold to penetrate the membrane, and to produce a channel through which the A1 polypeptide could pass (Gill, 1976a). The results of photoaffinity labelling studies (see earlier in this section) are not consistent with this theory.
- (b) The channel for the A1 polypeptide may be composed of a complex of the B subunits and integral membrane proteins. This hypothesis is not supported by the results of photoaffinity labelling experiments (see earlier in this section).
- (c) There could be direct interactions between the A subunit and integral membrane proteins or lipids. Preincubation of macrophages with compounds which inhibit protein synthesis (puromycin and cycloheximide)

decreased the effect of cholera toxin on the cells. These agents did not inhibit the activation of adenylate cyclase by hormones, therefore it was concluded that the synthesis of protein was required for cholera toxin to act across the cell membrane (Hagmann & Fishman, 1981). This system needs further investigation.

The A1 polypeptide of cholera toxin may dissolve directly into the membrane

Treatment of intact cells with the A subunit from cholera toxin activated adenylate cyclase, but the A subunit was much less potent than the complete toxin (van Heyningen & King, 1975; Wodnar-Fillipowicz & Lai, 1976). Uptake of a single molecule of diphtheria toxin is sufficient to kill a cell (Yamaizumi *et al.*, 1978). After entering a cell, diphtheria toxin has this high potency because it is a catalytically active protein (see section 1.4). Cholera toxin is also an enzyme (see section 1.3.3.1), therefore it is possible that the uptake of few, or only one, molecule of the A1 polypeptide is sufficient to activate adenylate cyclase. After the B subunits of cholera toxin have bound to ganglioside GM1 in the membrane, the high local concentration of A subunit may be sufficient to induce direct uptake of small amounts of the A1 polypeptide. The internalization of these few molecules may be enough to activate adenylate cyclase. This model is very difficult to prove or refute.

Active fragments may exert the intracellular effect of cholera toxin

Cholera toxin was covalently cross-linked to produce several species which contained A1 polypeptide, A2 polypeptide and B subunits. These cross-linked molecules and native toxin had similar abilities to activate adenylate cyclase in intact pigeon erythrocytes. There was a lag phase between the binding of native, or modified, cholera toxin to the cells

and stimulation of adenylate cyclase (van Heyningen, 1977b). Presumably when cells were treated with modified cholera toxin, part of the A subunit protruded through the membrane. This would have occurred despite the covalent bonds between the A and B subunits. Apparently small fragments of the A1 polypeptide may exert all of the ability of the molecule to activate adenylate cyclase (Matuo et al., 1976; Lai et al., 1981a). These portions could protrude through the membrane and be cleaved off by intracellular proteases. This may explain the activity of covalently cross-linked cholera toxin. But no radioactivity was released from [^3H] cholera toxin when it activated adenylate cyclase in pigeon erythrocytes (van Heyningen & Tait, 1979). Therefore it is possible, but unlikely, that active fragments may be responsible for the action of cholera toxin across membranes.

Uptake of cholera toxin by endocytosis

Certain compounds (e.g. methylamine, ammonium chloride, chloroquine and dansyl cadaverine) inhibit internalisation of receptors and processing of lysosomes. Treatment of intact HeLa cells with these reagents decreased the activation of adenylate cyclase by cholera toxin. The compounds failed to block the effect of cholera toxin in preparations of plasma membranes which were made from the same cells (Lin & Taniuchi, 1980). The same chemicals caused a lengthening of the lag phase between the binding of cholera toxin to rat hepatocytes and the activation of adenylate cyclase in the cells. The rate of activation of the enzyme was also decreased by these agents (Houslay & Elliott, 1981). This suggested that the action of cholera toxin across the plasma membrane involves lysosomes.

Cholera toxin was labelled with colloidal gold. Electron microscopy showed that the conjugates were associated with non-coated invaginations of the membranes and then entered the cytosol (Montesano et al., 1982).

The physiological significance of endocytosis in the entry of cholera toxin into cells is uncertain. Experiments that involve treatment of intact cells with agents which affect processing of membranes or functioning of lysosomes yield inconclusive results as the system is very complicated. Chemicals may be accumulated in certain parts of the cell at high concentrations and may affect adenylate cyclase or the activation of the enzyme by cholera toxin.

Endocytosis, by itself, could not explain the transmembrane action of cholera toxin. After endocytosis, there would still be a membrane between the toxin and its site of action. It has been proposed that endocytosis and processing in lysosomes is involved in the action of diphtheria toxin across membranes. (The mechanism of action of diphtheria toxin is outlined in section 1.4.) The low pH in lysosomes apparently enhances both the formation of a transmembrane channel by the B fragment of diphtheria toxin, and the unfolding of the active A fragment to facilitate its entry into the cytosol (Sandvig & Olsnes, 1980; van Heyningen, 1981).

There is very little direct evidence regarding how cholera toxin exerts its effect across the plasma membrane. The nature of the transport process, and even of what is transported, remains elusive.

1.3.1.3 Hybrid toxins

If the B subunits of cholera toxin can induce the uptake of the A1 polypeptide, they may also do so for other proteins. A hybrid toxin was made from the B subunits of cholera toxin and the A fragment of diphtheria toxin. Unlike either of its constituents, this molecule inhibited protein synthesis in intact cells from several lines (which is a property of

complete diphtheria toxin (see section 1.4)) (Mannhalter et al., 1980). The active polypeptides from toxins could be coupled to molecules (e.g. monoclonal antibodies) which specifically recognise receptors (see Olsnes, 1981). Such hybrid molecules could be cytotoxic to certain cells (e.g. cancer cells) and not to others. The inhibition of one group of cells in a mixed culture was achieved by treating them with a hybrid molecule which was made by coupling the active fragment of diphtheria toxin, or of ricin, to specific antibodies (Oelmann & Forbes, 1981). The scope for the use of hybrid toxins as pharmacological and biochemical agents is very exciting.

1.3.2 The regulation of adenylate cyclase

1.3.2.1 The functional units of adenylate cyclase

Eukaryotic adenylate cyclase is a complex enzyme which is usually found only in the plasma membrane (Ross & Gilman, 1980). The enzyme has at least three functionally different units.

- (a) A hormone receptor which is found on the outer surface of the cell.
- (b) A regulatory unit which binds guanyl nucleotides.
(This protein is exposed to the inside of the cell.)
- (c) A catalytic unit which is located on the inner surface of the plasma membrane.

The mechanism of regulation of the catalytic unit is poorly understood. Complete separation of the components of adenylate cyclase and reconstitution to produce active holoenzyme has yet to be demonstrated. The ability to perform such an experiment would provide useful techniques to study how the catalytic unit is regulated.

In the current work, knowledge on the regulation of adenylate cyclase is reviewed with an emphasis on the enzyme in rat liver, as this was the experimental system that was used.

1.3.2.2 The regulation of adenylate cyclase by guanyl nucleotides

Guanyl nucleotides are required for the stimulation of adenylate cyclase in rat liver by hormones (Rodbell et al., 1971). In many systems guanyl nucleotides decreased the affinity of the receptor for its hormone. This interaction was not competitive (Ross & Gilman, 1980). Analogues of GTP which resist hydrolysis of the γ -phosphate (e.g. β , γ -imido guanosine 5'-triphosphate, p(NH)ppG) cause persistent activation of adenylate cyclase (Pfeuffer & Helmreich, 1975). The structures of several analogues of GTP which resist hydrolysis are given in fig. 1.4. When rat liver membranes were incubated at 25°C, there was a lag of 3.5min after treatment with p(NH)ppG before adenylate cyclase was activated. This lag was decreased or abolished by the addition of glucagon, suggesting that hormones stimulate the exchange of guanyl nucleotides at the regulatory site (Rodbell et al., 1975). Rat liver membranes bind p(NH)ppG very tightly, the apparent affinity constant measured by Rodbell et al., (1975) was 10^9 - 10^{10} M^{-1} .

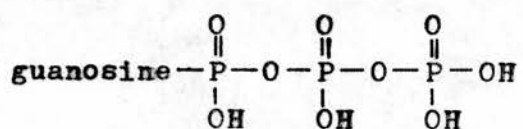
[^{14}C]p(NH)ppG was incubated with pigeon erythrocyte membranes which were then washed, solubilised and fractionated by gel-permeation chromatography. The radiolabel eluted with a protein of apparent $M_r = 230\,000$ (Pfeuffer & Helmreich, 1975). A membrane protein that bound guanyl nucleotides was also isolated by affinity chromatography on GTP γ S-agarose (fig. 1.4) (Pfeuffer & Helmreich, 1975). A protein which binds GTP has been partially purified from plasma membranes from many sources. The protein, or one of its constituent polypeptides, has a $M_r = 42\,000$ (e.g. see Pfeuffer, 1979; Cassel & Pfeuffer, 1978; Ross & Gilman, 1980).

Fig. 1.4

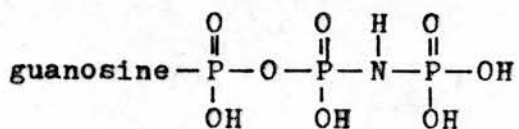
The structures of GTP and its hydrolysis-resistant analogues

The use of these compounds is discussed in section 1.3.2.2.

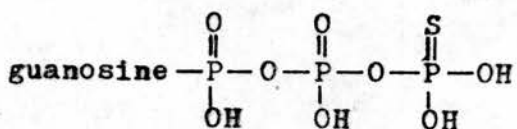
guanosine 5'-triphosphate, GTP



β,γ -imidoguanosine 5'-triphosphate, p(NH)ppG



guanosine 5'-(γ -thio)triphosphate, GTP γ S



In some preparations of plasma membranes hormones were shown to stimulate both adenylate cyclase and GTPase activities (Cassel et al., 1977; Levinson & Blume, 1977). When the hormone is released from its receptor the GTPase activity returns to a low level. The hormone-regulated GTPase failed to release the GDP which it produced until the enzyme was activated again (Cassel & Selinger, 1978).

Cholera toxin has been used extensively to study the regulatory component of adenylate cyclase (see section 1.3.3.2).

Fluoride ions are very potent stimulators of adenylate cyclase. The mechanism by which they activate the enzyme is not known, but they do act through the same regulatory protein as the guanyl nucleotides (Ross & Gilman, 1980).

Magnesium ions change the activity of adenylate cyclase. The enzyme is inhibited by nonchelated nucleotides (Rodbell et al., 1975), but the involvement of Mg^{2+} is complicated (Cech et al., 1980).

1.3.2.3 Models for the regulation of adenylate cyclase

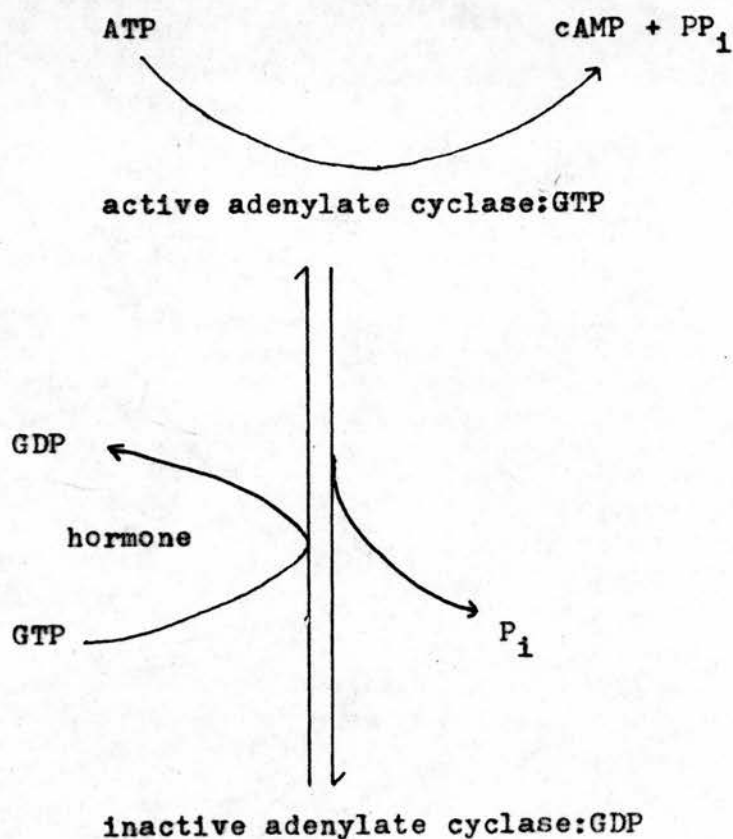
Many theories have been proposed to explain how the activity of adenylate cyclase is modulated by hormones and guanyl nucleotides. The most clearly understood system is the regulation of catecholamine-stimulated enzyme in avian erythrocytes. On the basis of the results that are described above, the following mechanism was proposed for the regulation of adenylate cyclase activity (Cassel & Pfeuffer, 1978; Cassel & Selinger, 1978; Cassel et al., 1979) (fig. 1.5).

The catalytic unit normally exists in a form which has a low activity. The binding of hormone to its receptor induces displacement of GDP from the regulatory component (which has GTPase activity). GTP then binds to the regulatory protein to induce increased activity in the catalytic unit, and decreased affinity for the hormone in the receptor. The

Fig. 1.5

The mechanism of regulation of adenylate cyclase in avian erythrocytes

This model is explained in the text (section 1.3.2.3).



P_i=inorganic phosphate

PP₁=inorganic pyrophosphate

regulatory component then hydrolyses the GTP to inorganic phosphate and GDP, the latter product remaining bound to the protein. When GDP is bound to the regulatory component, the catalytic unit has a low activity and the receptor has a high affinity for its hormone.

This model explains only the functional interactions between the constituents of adenylate cyclase, it does not suggest what physical interactions occur. Martin et al., (1979, 1980) used radiation-inactivation to study the physical interactions of the components of adenylate cyclase in rat liver.

Determination of molecular size by radiation-inactivation

Exposure of molecules to high-energy radiation results in loss of their function. This is because each primary ionisation deposits large amounts of energy (about 1 500 kcal/mol). Conditions are chosen such that ionisation is random, therefore the probability of zero hits on a molecule decreases exponentially with increasing volume of the target, or with increasing dose of radiation. Radiation-inactivation is used to determine the volumes of the structures that are required for a specific function. The size that is observed (which is called the functional unit), is an estimate of the minimum volume of the structures. The source of ionising radiation is generally a linear accelerator. The samples are usually irradiated below -110°C in a cooling bath of liquid nitrogen. The use of radiation-inactivation to determine the size of enzymes was reviewed by Kempner and Schlegel (1979).

The technique was useful in studying adenylate cyclase as it can measure target-sizes in an impure preparation in a membrane. However experiments are complicated by the existence of separate components of the enzyme. The effects of radiation on other factors which may be involved in the regulation of adenylate cyclase are unclear. Such factors may include lipids in the membrane (Levitzki, 1981) and

cytoskeletal elements (Sahyoun et al., 1981b). Freezing to -110°C and subsequent thawing may damage the system which regulates adenylate cyclase.

Alternative models for the regulation of adenylate cyclase

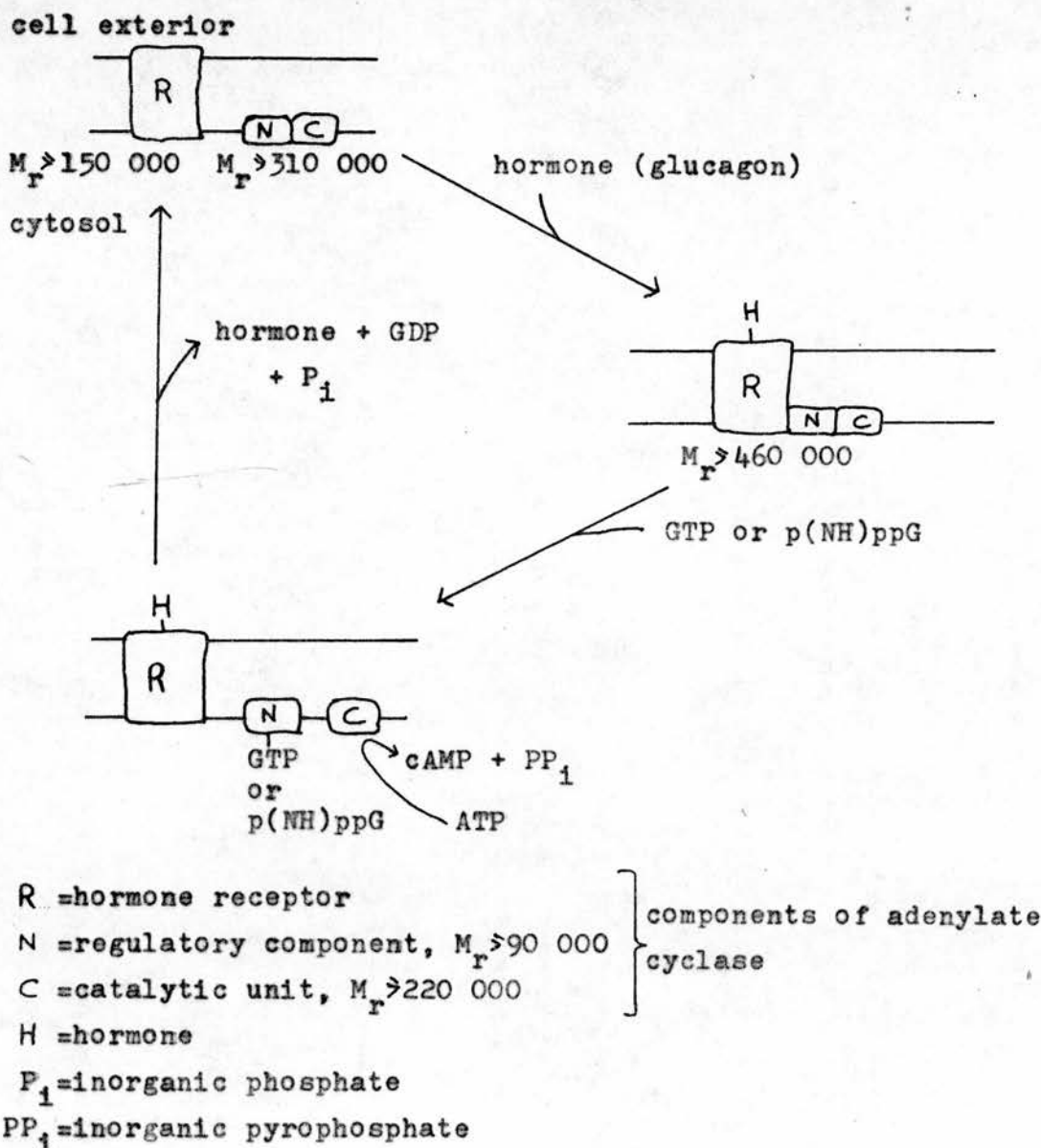
Several groups have used radiation-inactivation to study changes in the aggregation of the components of adenylate cyclase (e.g. see Rodbell, 1980; Martin et al., 1979, 1980). There is disagreement between these groups, but Martin and his colleagues have proposed a convincing model for the physical interaction of the constituents of adenylate cyclase in rat liver (fig. 1.6). The model is fairly consistent with that proposed for the functional interaction of the components of adenylate cyclase in avian erythrocytes (see above). Martin's model proposes that the catalytic and regulatory components usually exist as aggregations containing one molecule of each protein. The hormone receptor is free in the membrane. The binding of hormone results in the formation of a complex containing one molecule of each of the three types of constituents of the enzyme. The regulatory protein then binds GTP, and this causes the complex to break down into its constituent proteins. Then the catalytic factor becomes activated and the regulatory component exhibits GTPase activity. Hydrolysis of the GTP is followed by re-association of the regulatory and catalytic units. The coming together of these components returns the activity of the catalytic protein to a low level.

Other workers used kinetic analysis to study the mechanism of regulation of adenylate cyclase (Levitzki, 1981; Tolkovsky et al., 1982). Any GTPase that may regulate adenylate cyclase in rat liver has not been studied on a kinetic basis because plasma membranes are contaminated with other GTPases that are not associated with adenylate cyclase (Doberska et al., 1980). But the theory behind the use of kinetics to study the interactions of the components of adenylate cyclase fails to consider the following points.

Fig. 1.6

A model for the interaction of the components of adenylate cyclase in rat liver

This model was proposed by Martin et al. (1979, 1980) (see section 1.3.2.3). M_r values for the components of adenylate cyclase were determined using radiation-inactivation.



- (a) Models rarely explain, nor allow for, the GTP-induced decrease in affinity of the receptor for its hormone (Ross & Gilman, 1980).
- (b) Models ignore the fact that basal adenylate cyclase activity may result from spontaneous exchange of guanyl nucleotides, or from catalytic activity when the regulatory protein is binding GTP, or from a total of both.

Studying the kinetics of the regulation of adenylate cyclase can only characterise the rate-limiting step in the activation process. Such experiments showed that the hormone receptor is important in determining the rate of stimulation of adenylate cyclase, but not the final activity that is reached (Levitzki, 1981; Tolkovsky *et al.*, 1982). This is presumably because the binding of hormone stimulates exchange of guanyl nucleotides at the regulatory site. The observation is therefore consistent with both of the models for the regulation of adenylate cyclase that were discussed earlier in this section (see figs. 1.5 and 1.6).

Proteins in the cytosol may regulate adenylate cyclase (e.g. see Katz *et al.*, 1978 ; Crawford *et al.*, 1980), but the physiological significance of these molecules is uncertain (Ross & Gilman, 1980). The cytosolic location of these proteins may be artefactual. They may have detached from the plasma membrane during purification (Schleifer *et al.*, 1982; Pinkett & Anderson, 1982).

The properties of adenylate cyclase are poorly understood. There is a lack of knowledge about the interactions of the components of the enzyme, and the flow of regulatory activity between them.

1.3.3 The mechanism of activation of adenylate cyclase by cholera toxin

Intravenous injection of cholera toxin activated adenylate

cyclase in rat liver (Flores & Sharp, 1975). In preparations of broken cells NAD^+ is required for the stimulation of adenylate cyclase by cholera toxin (Gill, 1975). Presumably cytosolic NAD^+ fulfils this requirement in intact cells.

1.3.3.1 The enzymic activity of cholera toxin

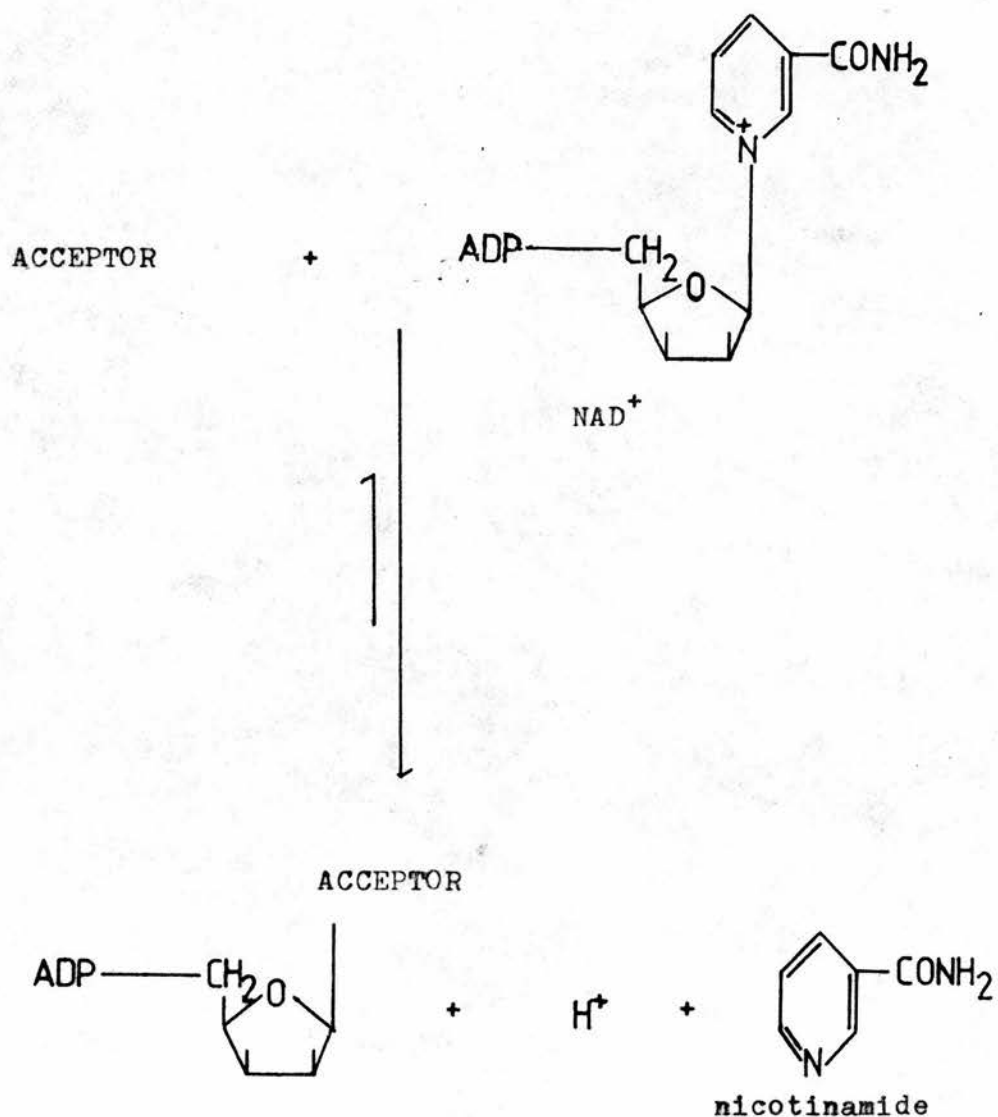
Preparations of cholera toxin catalysed the release of nicotinamide from NAD^+ . This activity was greatly enhanced by the addition of arginine methyl ester (which is an analogue of arginine residues in proteins). The production of nicotinamide was increased to a lesser extent in the presence of arginine in place of arginine methyl ester. Citrulline failed to affect the reaction (Moss & Vaughan, 1977). Citrulline is similar in structure to arginine, having a ureido group rather than a guanidino group. Incubation of $[\text{adenosine-}^{14}\text{C}] \text{NAD}^+$ and $[\text{H}^3] \text{arginine}$ in the presence of cholera toxin produced a compound that was isolated by thin-layer chromatography. The product contained ^3H and ^{14}C in equimolar amounts (Moss & Vaughan, 1977). These results suggested that cholera toxin could transfer ADP-ribose from NAD^+ to arginine.

Incubation of hepatocytes with cholera toxin and $[\text{adenine-}^3\text{H}] \text{NAD}^+$ resulted in the labelling of material which was precipitated by trichloroacetic acid (TCA). When the radiolabel was replaced by $[\text{nicotinamide-}^{14}\text{C}] \text{NAD}^+$ there was no incorporation of counts into TCA-precipitable material. With $[\text{adenine-}^3\text{H}] \text{NAD}^+$ the amount of radiolabel that was precipitated by TCA correlated roughly with the stimulation of adenylate cyclase by cholera toxin. The radiolabelled molecules were sensitive to trypsin and were therefore thought to be proteins. The labelling of the proteins was stable to dilute acid, but not to 1M NaOH, implying that the modifying group was ADP-ribose linked through the terminal ribose (fig. 1.7) (Beckner & Blecher, 1978).

Fig. 1.7

NAD⁺-dependant ADP-ribosylation

ADP-ribose is transferred from NAD⁺ to an acceptor as shown. In glycohydrolysis of NAD⁺ the acceptor is the hydroxyl group of water. The role of ADP-ribosylation in the action of cholera toxin is discussed in section 1.3.3.



Membranes from pigeon erythrocytes were incubated with [γ -adenylate- ^{32}P] NAD^+ and cholera toxin, washed and then boiled in 0.1M HCl. Radiolabelled compounds were released that comigrated with ADP-ribose and AMP on thin-layer chromatographs. This showed that cholera toxin was catalyzing ADP-ribosylation. AMP presumably arose from cleavage of the pyrophosphate bond of ADP-ribose (Cassel & Pfeuffer, 1978).

Cholera toxin catalyses a reaction between NAD^+ and arginine. Nuclear magnetic resonance spectroscopy showed that the product was ADP-ribosyl arginine (Oppenheimer, 1978). This conclusively proved that cholera toxin is an ADP-ribosyl transferase.

In vitro, cholera toxin catalyzed the ADP-ribosylation of several proteins (Trepel et al., 1981). High concentrations of substrate proteins were used, therefore these results have little relevance to the situation in vivo, where the reaction seems to be more specific (see section 1.3.3.2). Cholera toxin ADP-ribosylates itself (Trepel et al., 1977). Several ADP-ribose residues may be incorporated onto the A1 polypeptide of the toxin. ADP-ribosylation increased the enzymic activity of the A1 polypeptide (Moss et al., 1980 a).

Some workers claim that cholera toxin has NAD^+ -glycohydrolase (NADase) activity (E.C. 3.2.2.5) as well as ADP-ribosyl transferase activity (fig. 1.7). Moss et al (1976 b, 1979 a) failed to separate NADase activity from cholera toxin or its A1 polypeptide using gel-permeation chromatography. Nor could the activity be separated from the A subunit by polyacrylamide-gel electrophoresis (PAGE). Tait and van Heyningen (1978) used PAGE to separate the NADase activity, but not the ability to activate adenylate cyclase, from cholera toxin. Only the NADase activity was inhibited by SDS. It was therefore suggested that, as the ADP-ribosyl transferase activity of cholera toxin produced activation of adenylate cyclase (see section 1.3.3.2), preparations of

cholera toxin were contaminated with NADase. This hypothesis was supported by the results of Fernandes et al (1979) who used purified A subunit of cholera toxin to raise antibodies. Treatment of preparations of cholera toxin with these antibodies decreased their ADP-ribosyl transferase activity but did not affect their capacity to hydrolyze NAD^+ .

1.3.3.2 The substrates of cholera toxin in membranes and their roles in the regulation of adenylate cyclase

After stimulation by the hydrolysis-resistant analogue of GTP, p(NH)ppG , adenylate cyclase from rat liver could not be further activated by cholera toxin (Flores et al., 1976). Cholera toxin induced an increase in basal and hormone-stimulated adenylate cyclase activities in turkey erythrocytes. The toxin also inhibited the GTPase activity that was stimulated by catecholamines (see section 1.3.2) (Cassel & Selinger, 1977; Cassel et al., 1977). Blocking the GTPase activity with p(NH)ppG prevented further inhibition of the enzyme by cholera toxin. Therefore the two stimulators did not have additive effects on the activity of adenylate cyclase.

In membranes prepared from turkey erythrocytes NAD^+ was required for both the inhibition of GTPase, and the stimulation of adenylate cyclase, by cholera toxin (Cassel & Selinger, 1977; Cassel et al., 1977). Cholera toxin catalyzed the transfer of radiolabel from $\text{[adenylate-}^{32}\text{P] NAD}^+$ to several proteins in pigeon erythrocyte membranes. Most of the radioactivity was in a polypeptide of $M_r = 42\ 000$. This polypeptide (or a molecule containing it) was solubilized with a non-ionic detergent and then partially purified using a GTP-Sepharose affinity column. Addition of this protein to control membranes (which had not been treated with cholera toxin) enhanced the ability of GTP to stimulate adenylate cyclase (see section 1.3.2) (Cassel & Pfeuffer, 1978). At low pH and in the presence of nicotinamide, radiolabelling of the protein was partially reversed by cholera toxin. This

suggested that the equilibrium of an ADP-ribosylation reaction was being shifted by the products (fig. 1.7) (Cassel & Pfeuffer, 1978). Cholera toxin transfers radioactivity from [adenylate- ^{32}P] NAD^+ to a polypeptide of $M_r \simeq 42\,000$ in many systems, and to additional polypeptides in some systems (see Johnson *et al.*, 1980; Johnson, 1982).

Experiments to obtain further evidence supporting the idea that cholera toxin ADP-ribosylates the regulatory component of adenylylase have involved reconstitution or complementation of the enzyme. Mutants derived from the mouse S49 lymphoma cell-line have been used in most of these studies. The characteristics of some of these mutants are given in table 1.1.

Detergent was used to make an extract from wild-type S49 membranes. This extract reconstituted sensitivity to p(NH)ppG in adenylylase in membranes from S49 cyc^- cells (Johnson *et al.*, 1978). This showed that regulatory protein could be solubilized from one set of membranes to activate the catalytic unit in another set of membranes. Wild-type S49 membranes were pretreated with cholera toxin, washed and then solubilized with detergent. When these extracts were used to reconstitute adenylylase in S49 cyc^- membranes, the enzyme which was produced was equally sensitive to stimulation by GTP or p(NH)ppG . If membranes had not been pretreated with cholera toxin, GTP had little or no effect on the activity of the reconstituted enzyme (Johnson *et al.*, 1978). Donor extracts from S49 cyc^- membranes that had been pretreated with cholera toxin reconstituted adenylylase which was not sensitive to GTP (Johnson *et al.*, 1978). The results of these experiments confirmed the hypothesis that cholera toxin modifies the regulatory component of adenylylase to inhibit its GTPase activity.

The mechanism activation of adenylylase by cholera toxin needs further investigation (see sections 1.5 and 6.3).

Table 1.1

Characteristics of membranes used for complementation analysis of adenylate cyclase

The theoretical basis of complementation experiments is given in section 1.3.3.2. The S49 cell-line is derived from a mouse lymphoma. Phenotypes refer to the components of adenylate cyclase (R = hormone receptor, C = catalytic unit, N = regulatory protein).

	wild-type S49	S49 cyc ⁻	avian erythrocytes
<u>phenotype</u>	R ⁺ N ⁺ C ⁺	R ⁺ N ⁻ C ⁺	R ⁺ N ⁺ C ⁺
<u>activation of adenylate cyclase by:</u>			
isoproterenol	+	-	+
prostaglandin E ₁	+	-	-
cholera toxin	+	-	+
fluoride ions	+	-	+
<u>substrates of cholera toxin:</u>			
M _r = 42 000	+	-	+
M _r = 52 000	+	-	-
<u>reconstitution ability with S49 cyc⁻:</u>			
stimulation by hormones	+	-	+
stimulation by fluoride ions	+	-	+
stimulation by GTP γ S	+	-	+
stimulation by cholera toxin	+	-	+

Most experiments have been performed on cancer cells or avian erythrocytes. Such systems have unusual biochemical properties. The mechanism of action of cholera toxin should also be investigated in systems that are more conventional. Cholera toxin can be used to study how adenylate cyclase is regulated (see sections 4.3 and 6.2).

1.4 SIMILARITIES BETWEEN CHOLERA TOXIN AND OTHER MOLECULES

(a) Heat-labile toxin from *Escherichia coli*

Some strains of *E.coli* cause a diarrhoea which is more mild than cholera. The production of the disease by *E.coli* is due to secretion of a toxin. *E.coli* produce many toxic proteins, but the induction of diarrhoea by *E.coli* occurs because a heat-sensitive toxin (which is called labile toxin or LT), activates adenylate cyclase (Evans et al., 1972). LT and cholera toxin are the same enzyme, but they show slight differences that are associated with their origins from different species. The two toxins have similar receptors on the host cell surface (Moss et al., 1979b), structures (Spicer et al., 1981; Dallas & Falkow, 1980; Gill et al., 1981) and mechanisms of action (Moss & Vaughan, 1981).

(b) Diphtheria toxin

The pathogenesis of diphtheria is dependant upon the production of a toxic protein. Diphtheria toxin is composed of a catalytically active (A) fragment and a B fragment that is responsible for binding to susceptible cells and inducing uptake of the A fragment (Pappenheimer & Gill, 1977). Diphtheria toxin blocks protein synthesis at 80S ribosomes by ADP-ribosylating elongation factor 2 (EF-2) to inhibit its GTPase activity (Honjo et al., 1969). The mechanism of action of diphtheria toxin is therefore similar to that of cholera toxin, but the two toxins do not have

similar amino-acid sequences (see van Heyningen, 1980). Diphtheria toxin ADP-ribosylates a modified histidine residue, named diphthamide (Van Ness et al., 1980). This may be important in determining the specificity of ADP-ribosylation by the toxin (see section 5.3.3).

Exotoxin A produced by Pseudomonas aeruginosa is very similar to diphtheria toxin: it also ADP-ribosylates EF-2 to inhibit its GTPase activity (Inglewski et al., 1977).

(c) Mono(ADP-ribosyl) transferases in eukaryotic cells

The attachment of long chains of ADP-ribose residues to proteins in the nucleus has been extensively studied (Ueda et al., 1979). Avian erythrocytes contain an enzyme which transfers mono(ADP-ribose) residues to small molecules containing guanidinium groups. The enzyme also ADP-ribosylates proteins (Moss et al., 1980b). Several sources contain enzymes which have similar properties (for example see Moss & Stanley, 1981). Mono(ADP-ribosyl) transferase activity has been found in rat liver (Kun et al., 1975; Moss & Stanley, 1981). It has been suggested that ADP-ribosylation is involved in the regulation of adenylate cyclase in healthy cells.

ADP-ribosylation seems to be involved in the regulation of adenylate cyclase in rat mammary tumours (Cho-Chung et al., 1980), rat hepatoma cells (Beckner et al., 1980 ; Reilly et al., 1981) and thyroid tissue (Vitti et al., 1982). The role of ADP-ribosylation is very unclear. Hormones apparently activate an ADP-ribosyl transferase which is in the membranes of the target cells. ADP-ribosylation seems to decrease the sensitivity of adenylate cyclase to subsequent stimulation by hormones (de Wolf et al., 1981).

1.5 AIMS OF THE PROJECT

The mechanism of action of cholera toxin was investigated in a system where its substrates could be radiolabelled in sufficient amounts to study their structures and functions. Rat liver was chosen as the experimental system for reasons which are given in section 2.1. The objectives could be summarised as follows.

- (a) To measure the hydrophobicities of cholera toxin and its subunits, and to relate the results to the mechanism of the action of cholera toxin across membranes.
- (b) To examine the mechanism of activation of adenylate cyclase by cholera toxin.
- (c) To study the effects of activation on the structure and function of adenylate cyclase.
- (d) To investigate the structures of the polypeptides which are substrates of cholera toxin.

2. GENERAL METHODS

2.1 The experimental system

2.1.1 Choice of experimental system

Membranes which contain adenylate cyclase are easily prepared from rat liver. Adenylate cyclase in rat liver has been thoroughly investigated (e.g. see reviews by Rodbell et al., + 1975; Abramowitz et al., 1979). Enzymes which degrade NAD are also well-studied (Nakazawa et al., 1968; Bock et al., 1971; Tait, 1980).

In this laboratory Tait (1980) developed a system in which cholera toxin is active at very low concentrations of NAD^+ . This enables the use of NAD^+ at high specific radioactivity, as is required for the labelling of molecules by cholera toxin. However this preparation does contain many intracellular membranes (see section 2.1.3) apart from the plasma membranes where adenylate cyclase is located (Wattiaux-De Coninck et al., 1981).

Adenylate cyclase is very labile, which makes purification difficult, possibly due to the loss of regulatory factors (Katz et al., 1978). This may explain why the specific activity of adenylate cyclase in crude preparations is similar to that found in plasma membranes (table 2.1). Purified plasma membranes could be heavily contaminated with denatured enzyme which may confuse studies on the mechanism of action of cholera toxin. To purify the enzyme (rather than the membranes in which it occurs), solubilization is required, but this also causes marked loss of activity (table 2.1).

Table 2.1

The effects of different preparation procedures on the activity of
adenylate cyclase from rat liver

The experimental procedures for the current work are given in sections 2.1 and 2.2.

<u>preparation and</u>	<u>adenylate cyclase</u>	<u>reference</u>
<u>incubation temperature</u>	<u>activity</u>	
	<u>(pmol cAMP produced/ min/mg protein)</u>	
	<u>basal</u>	<u>cholera toxin-</u> <u>stimulated</u>
<u>crude membranes</u>		
25°C	28	70
37°C	18	56
25°C	10-20	20-50
		Tait (1980)
		Flores <u>et al.</u> , (1976)
		Current work (sections 2.2 and 4.2)
<u>purified plasma membranes</u>		
30°C	26	59
30°C	35	64
		Martin <u>et al.</u> , (1977)
		Doberska <u>et al.</u> , (1980)
<u>solubilized enzyme</u>		
15°C	0.8	4.3
15°C	6.4	17.6
		van Heyningen (1976b)
		Current work (tables 4.9 and 4.10)

The information which is given in this table is discussed in section 2.1.1.

Having considered all of these factors it was decided that the system which was developed by Tait (1980) was optimal for the present work.

2.1.2 Preparation of rat liver membranes

The liver of a freshly killed female Wistar rat was perfused through the portal vein with ice-cold 10mM HEPES containing 0.25M sucrose and 5mM MgCl_2 , adjusted to pH7.4 with NaOH. The liver was washed in the same buffer then homogenised (5cm^3 buffer: 2g liver) using ten strokes with a teflon pestle in a glass homogeniser. The homogenate was filtered through one layer of surgical gauze then centrifuged at room temperature and $3\,500\text{ g}_{\text{max}}$ for 5 minutes. The pellet was washed twice then resuspended in an equal volume of perfusion buffer and stored on ice for use the same day. Long term storage of membranes was at -70°C .

2.1.3 Characterisation of the preparation of rat liver membranes

Marker enzymes and DNA were assayed as outlined in table 2.2. The composition of the preparation of rat liver membranes was compared to that of homogenised rat liver (fig. 2.1).

Trypan blue is a dye which enters broken cells but not intact cells (Evans, 1978). Observation under a light microscope showed that there were no structures in the homogenate, nor in the membrane preparation, which excluded trypan blue. Therefore there was free access for substrates and cofactors in studies on the action of cholera toxin and in assays of adenylate cyclase activity. This result is consistent with the rapid activation of adenylate cyclase in the membrane preparation by cholera toxin as the 20 min-incubation used in this work would probably not have been long enough to allow for the lag phase which is seen with intact cells (see section 1.3.1.1). Also the A subunit of cholera toxin potently activated adenylate cyclase (fig. 2.5) indicating that the cells must be broken.

Table 2.2

Assays for marker enzymes and DNA

<u>marker</u>	<u>subcellular localisation</u>	<u>basis of assay</u>	<u>references</u>
protein	none	increase in E_{595} of Coomassie Brilliant Blue when it binds to protein	Bradford (1976)
DNA	nucleus (mitochondria)	DNA isolated then reacted with diphenylamine. Products measured spectrophoto- metrically	Burton (1956) Evans (1978)
glucose-6- phosphatase	endoplasmic reticulum	phosphate released from glucose-6- phosphate measured as a blue phospho- molybdate complex	Evans (1978) Bell & Doisy (1920)
cytochrome oxidase	mitochondria	cytochrome c reduced with dithionite. Oxidation by sample followed using E_{550}	Mason <u>et al.</u> , (1973) Evans (1978)
adenylate cyclase	plasma membrane	cAMP produced from γ - ^{32}P ATP, then purified and counted	Current work (section 2.2), Evans (1978)

(table 2.2 continued on page 37)

Table 2.2 (continued)

<u>marker</u>	<u>subcellular localisation</u>	<u>basis of assay</u>	<u>reference</u>
β -glucuronidase	lysosomes	release of phenolphthalein from phenolphthal- ein glucuronide followed by E ₅₅₀	Evans (1978)

Experiments using these assays are described in section 2.1.3.

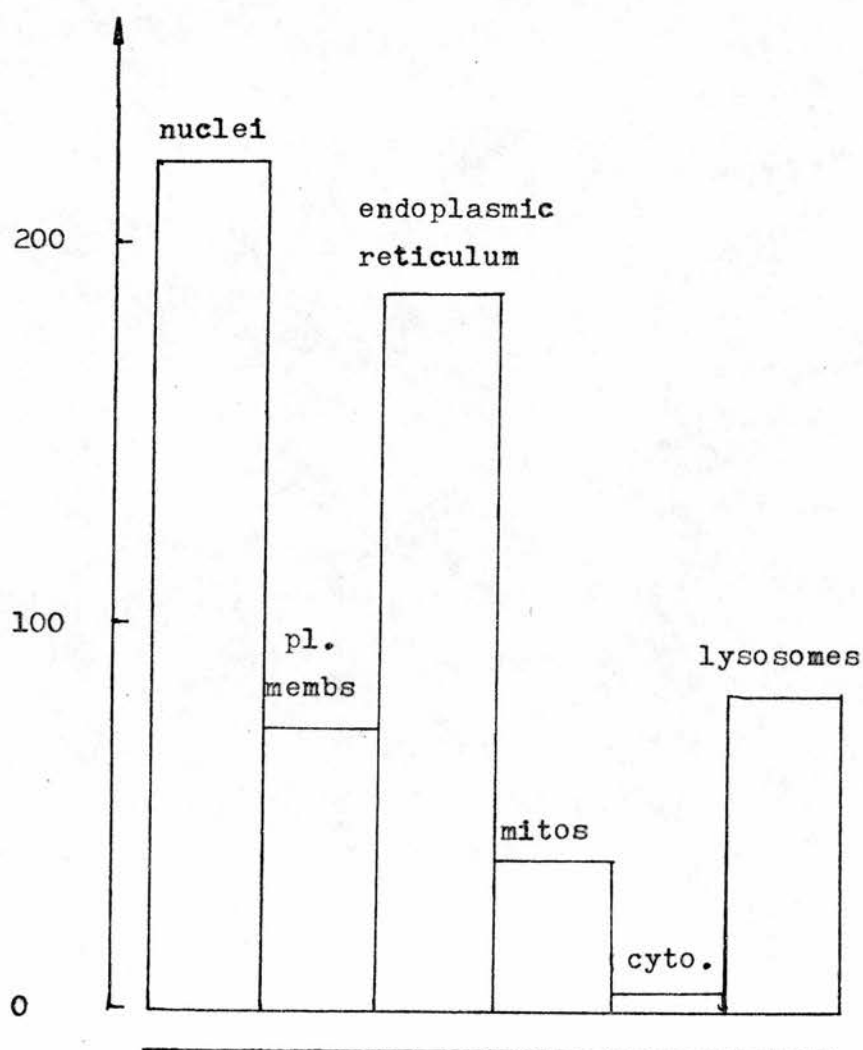
Fig. 2.1

Composition of the rat liver membrane preparation relative to homogenate

Rat liver membranes and liver homogenate were prepared in the same buffer and then marker enzymes and DNA were assayed (see section 2.1). Similar results were obtained when the experiment was repeated.

pl. membs = plasma membranes, mitos = mitochondria, cyto. = cytosol.

amount in membrane preparation
per mg protein (% relative to
homogenate)



The preparation of membranes contained most of the organelles from the cell, but was depleted in mitochondria and enriched in nuclei and endoplasmic reticulum. Cytosol was almost completely removed but about 75% of the plasma membranes were retained (this figure was not corrected for possible lability of the marker enzyme) (fig. 2.1).

2.1.4 Incubation of membranes from rat liver with cholera toxin

Cholera toxin at $300-400 \mu\text{g cm}^{-3}$ was preactivated by incubating at 25°C for 30 minutes with 0.01% (w/v) SDS, 7mM DTT and 3mg cm^{-3} BSA. This procedure apparently enhances the release of the A1 polypeptide from the rest of the toxin to increase its potency in affecting adenylate cyclase (van Heyningen, 1976a; Gill, 1976b). A modified version of the above mixture which did not contain cholera toxin was used for controls.

Freshly prepared membranes were incubated with cholera toxin for 20 minutes at 25°C . The other components of the mixture are given in table 2.3.

The incubation was stopped by addition of excess ice-cold 60mM MOPS adjusted to pH7.2 with NaOH, followed by vortex-mixing.

The rat liver membranes contain four major enzymes which degrade NAD^{+} . These are poly(ADP-ribosyl) polymerase, chromatin NADase (Nakazawa *et al.*, 1968), plasmalemmal NADase and microsomal NADase (Bock *et al.*, 1971). 1mM nicotinamide together with 20mM thymidine inhibited these enzymes without significantly affecting the action of cholera toxin (Tait, 1980).

This incubation allows the activation of adenylate cyclase by $5\mu\text{g cm}^{-3}$ cholera toxin to reach completion (fig. 2.4).

Table 2.3

Components of the mixture for the incubation of cholera toxin with rat liver membranes

membranes	10-15 μ g protein cm^{-3}
cholera toxin	10 μ g cm^{-3}
NAD ⁺	0.1mM
GTP	0.15mM
ATP	1.5mM
MgCl ₂	5mM
DTT	2mM
nicotinamide	1mM
thymidine	20mM
sucrose	0.25M
HEPES	10mM
NaOH	to pH7.4

This incubation mixture was used as described in section 2.1.4.

2.2 Assay of adenylate cyclase activity

2.2.1 Assay procedure

This technique is a modification of that of Salamon (1979).

After incubation with cholera toxin, rat liver membranes were washed with ice-cold 60mM MOPS which had been adjusted to pH7.2 with NaOH, then resuspended at about 4mg protein cm⁻³ in the same buffer. A 25µl sample was taken for incubation with an equal volume of assay cocktail (table 2.4). The assay cocktail was adjusted to pH7.2 with NaOH before addition of radioactive ATP and membranes. The stock radioactive ATP was at 10-50 Ci/mmol. Creatine kinase and creatine phosphate were used to regenerate nucleoside triphosphates. IBMX (Aldrich Chemical Co., Gillingham, Dorset, U.K.) is a potent inhibitor of cAMP phosphodiesterase (Peytreman *et al.*, 1973). The membranes were often preincubated with 20mM NaF then assayed for adenylate cyclase activity to test for functional adenylate cyclase and regulatory protein. Fluoride ions are potent activators of the enzyme (Ross and Gilman, 1980).

The incubation was carried out for 5 minutes at 25°C and was stopped by boiling for 3 minutes. A solution of 2% (w/v) SDS with 45mM ATP and 1.3mM cAMP was adjusted to pH7.5 with tris base. After the samples were boiled, 150µl of this solution was added to each incubation mixture to solubilize the membranes. The preparations were cooled on ice then 1cm³ double-distilled water and about 5 000 dpm [γ -³H]cAMP (30Ci/mmol) was added to each tube. This allowed estimation of the yield of [γ -³²P]cAMP after subsequent purification. (It was shown that cAMP is stable to boiling for 3 minutes.) These mixtures were chromatographed on Dowex AG 50W-X4 (200-400 mesh) columns and then neutral alumina WN-3 (Sigma London Chemical Co. Ltd, Poole, Dorset, U.K.) columns to separate [γ -³²P]cAMP from other compounds which were labelled with ³²P, as described by Salamon (1979). Mrs L. A. Rankine poured the columns and determined the elution volumes for cAMP and ATP. cAMP was completely separated from all other radioactive compounds, and the yield was 70-90%. The samples were counted for 10min

Table 2.4

Composition of the incubation used to measure activity of adenylate cyclase

rat liver membranes	about 2mg protein cm^{-3}
ATP (approximately $0.5\mu\text{Ci}$ α - ^{32}P ATP per assay)	0.5mM
GTP	0.01mM
MgCl_2	15mM
creatine phosphate	5mM
creatine kinase	50U cm^{-3}
DTT	2mM
IBMX	1mM
BSA	0.1mg cm^{-3}
MOPS	60mM

The activity of adenylate cyclase was assayed as described in section 2.2.1.

each on a Searle MK III Liquid Scintillation System which measured the quenching in each vial (see section 2.3). The results were corrected for cross-over between ^3H and ^{32}P by comparison with standards containing a single isotope.

In this work the cost of $[\text{}^{32}\text{P}]\text{ATP}$ limited the number of replicate adenylate cyclase assays to two in each case. The results are therefore quoted as means of duplicate determinations. For all of the values which are presented, the duplicates agreed to within $\pm 10\%$, and usually to within $\pm 5\%$. Experiments involving assay of adenylate cyclase were performed at least twice.

2.2.2 Characteristics of the assay

The production of cAMP by control and cholera toxin-treated membranes was linearly related to the amount of protein in the sample up to 0.12mg protein (fig. 2.2). 0.10mg protein was routinely taken for the assay of adenylate cyclase. cAMP production by cholera toxin-treated and control membranes was linear for 8min (fig. 2.3). Subsequent adenylate cyclase assays were incubated for 5min.

2.2.3 Characteristics of adenylate cyclase in rat liver membranes

Both cholera toxin and its A subunit activated adenylate cyclase in rat liver membranes. About $7\mu\text{g cm}^{-3}$ cholera toxin or $13\mu\text{g cm}^{-3}$ A subunit was sufficient to activate the enzyme maximally (figs 2.4 and 2.5).

The A subunit of cholera toxin was purified by Mrs L. A. Rankine using chromatography on Sephadex G-75 in urea at low pH (Moss *et al.*, 1976b). More A subunit than cholera toxin was required to activate adenylate cyclase maximally. This is surprising as the A subunit is the part of the toxin which is responsible for its stimulatory effect. The low potency of the A subunit may be due to the denaturation during separation and purification from the B subunits, and it may be because concentrations

Fig. 2.2

Production of cAMP by various amounts of rat liver membranes

Rat liver membranes were incubated with (■), or without (□) cholera toxin, washed and then assayed for adenylate cyclase activity using an incubation for ten minutes (see sections 2.1 and 2.2). Similar results were obtained when the experiment was repeated. Adenylate cyclase activities were assayed in duplicate. The replicates agreed to within $\pm 5\%$, and the mean values are quoted.

cAMP produced
(pmol/assay)

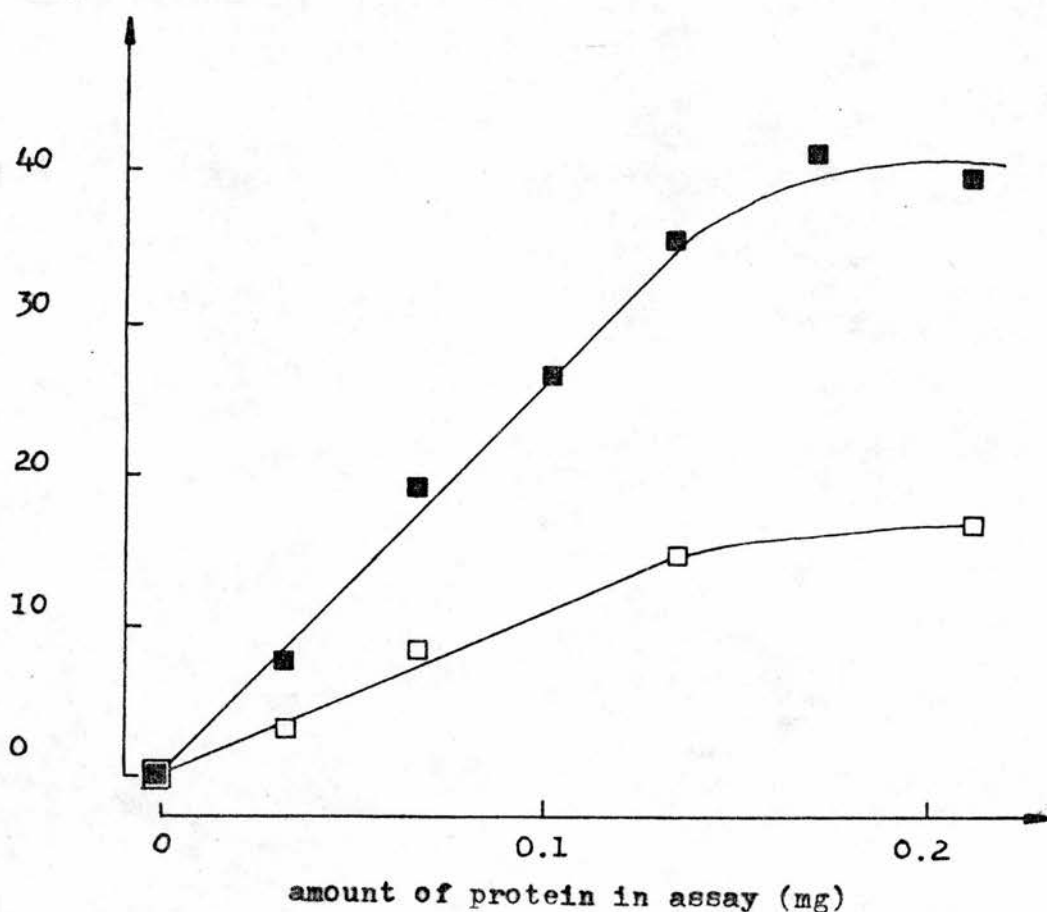


Fig. 2.3

Time-course of production of cAMP by rat liver membranes

Rat liver membranes were incubated with (■), or without (□) cholera toxin, washed and then assayed for adenylate cyclase activity (see sections 2.1 and 2.2). Similar results were obtained when the experiment was repeated. Adenylate cyclase activities were measured in duplicate. The replicates agreed to within $\pm 5\%$, and the mean values are quoted.

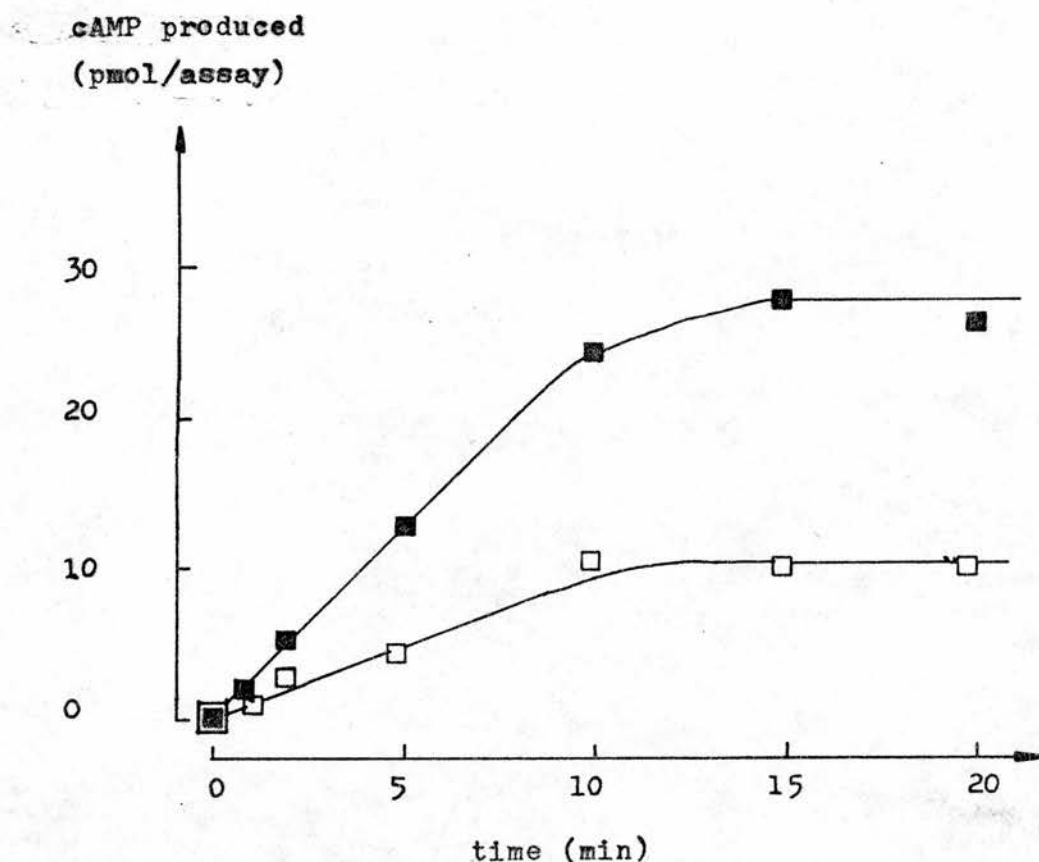


Fig. 2.4

Activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with various concentrations of cholera toxin, washed and then assayed for adenylate cyclase activity (see sections 2.1 and 2.2). The values quoted for adenylate cyclase activities are the means of duplicate determinations which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated.

adenylate cyclase activity
(pmol cAMP produced/min/
mg membrane protein)

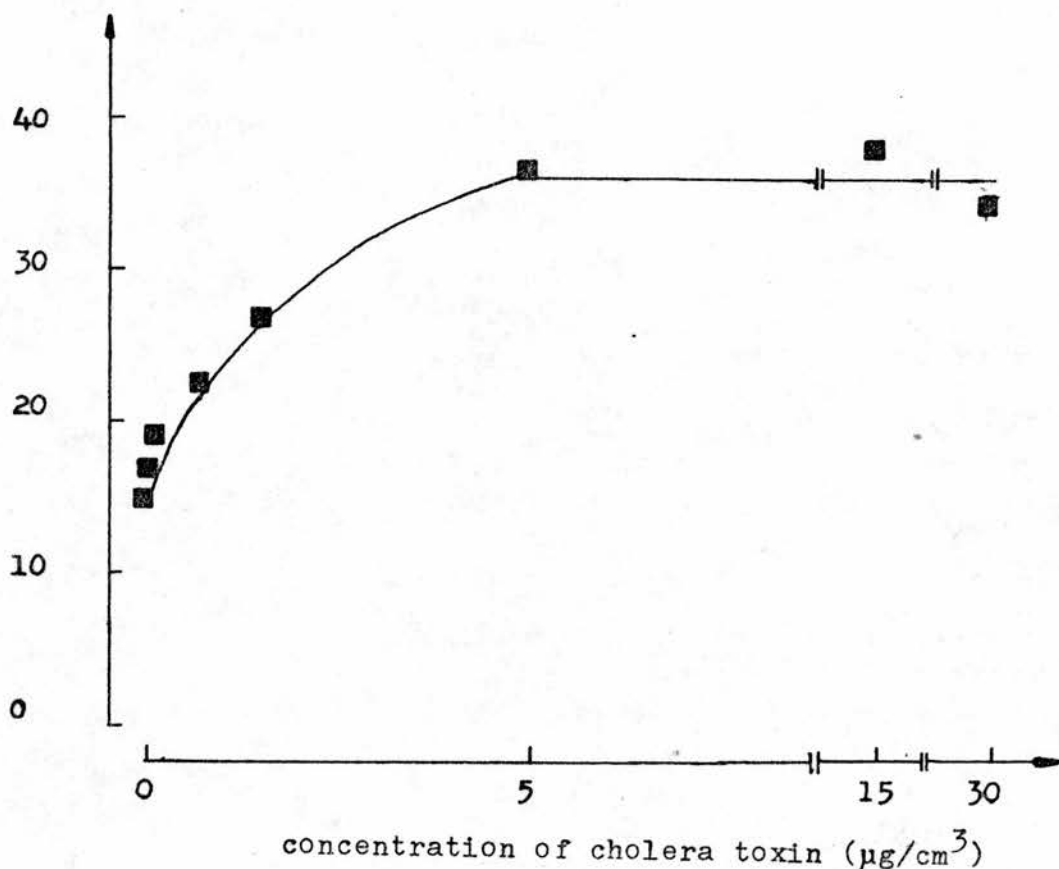
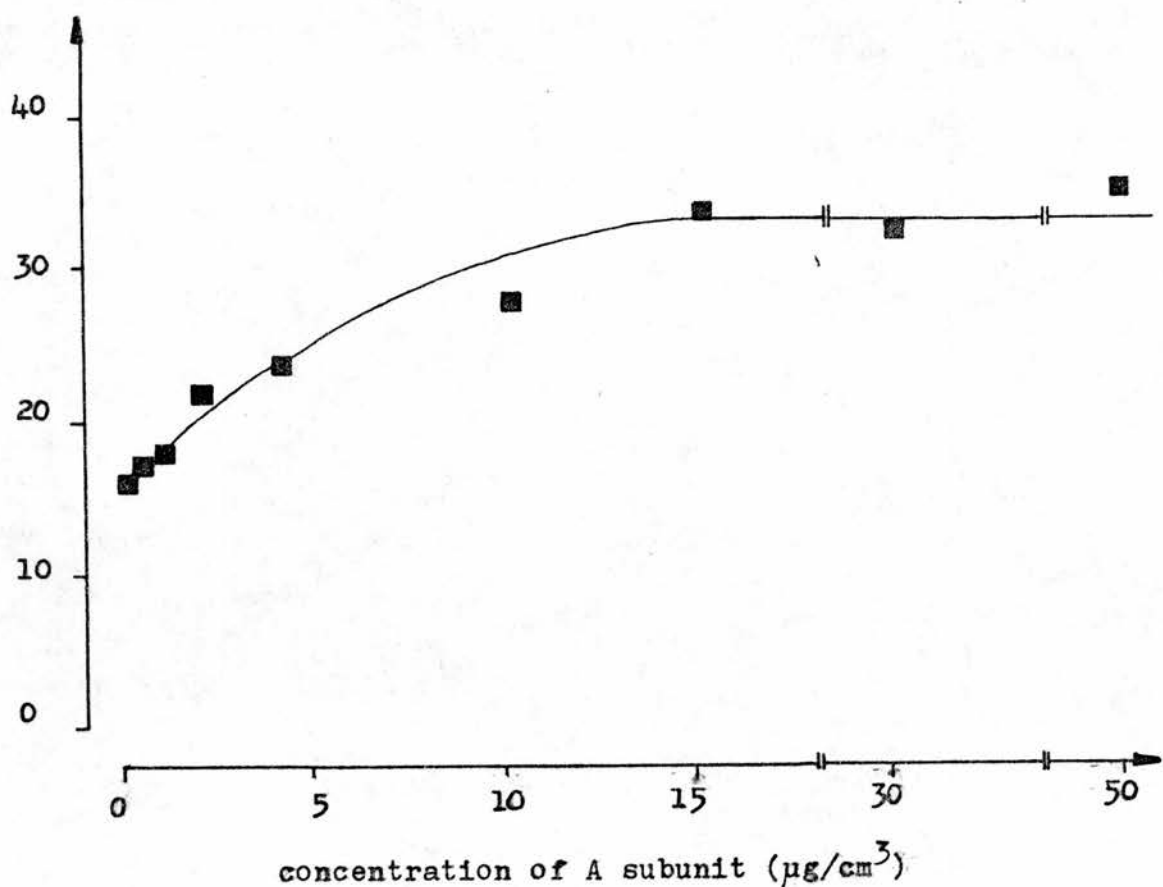


Fig. 2.5

Activation of adenylate cyclase by the A subunit of cholera toxin

Rat liver membranes were incubated with various concentrations of the A subunit from cholera toxin, washed and then assayed for adenylate cyclase activity (see sections 2.1 and 2.2). The values quoted for adenylate cyclase activities are the means of duplicate determinations which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated.

adenylate cyclase activity
(pmol cAMP produced/min/
mg membrane protein)



of A subunit were over-estimated. Estimates of the concentration of the A subunit of cholera toxin are often too high because the protomer is rich in tyrosine residues (Lai, 1980). This gives it an unusually high extinction coefficient at 280nm and also seems to affect the Bradford assay for protein (see section 2.4). Other workers have reported that the A subunit is apparently less potent than cholera toxin in stimulating adenylate cyclase even in preparations of broken cells (van Heyningen and King, 1975).

NaCl only had a small effect on basal and cholera toxin-stimulated adenylate cyclase activities. NaCl concentrations up to about 100mM increased basal and cholera toxin-stimulated adenylate cyclase activities. Above 100mM and up to about 250mM NaCl there was a slight decrease in activity which was possibly due to coagulation of the membranes. At very high levels (1.6M) of NaCl the effect of cholera toxin on adenylate cyclase activity could still be clearly seen (fig. 2.6). Similarly high concentrations of NaCl, nicotinamide or arginine methyl ester had little effect on the stimulation of adenylate cyclase by fluoride ions (fig. 2.7).

The ionic strengths in some of the incubations with cholera toxin could not be calculated accurately as they were so high that the solute behaviour would not be ideal. An estimate of ionic strength could be obtained by multiplying the concentration of NaCl by a factor of one, and the concentration of arginine methyl ester by 1.25. The factor for arginine methyl ester is based upon the dissociation constants given by Dawson et al., (1979). With respect to the activation of adenylate cyclase by high ionic strength, there was good agreement between the results for NaCl and arginine methyl ester (fig. 2.7). The effects of nicotinamide on the activity of adenylate cyclase are considered in section 4.2.3.1.

Other workers (Katz et al., 1980) have confirmed these results on the effect of high ionic strength on the activity of adenylate cyclase. The mechanism of these effects is unknown.

Fig. 2.6

The effect of NaCl on the activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with (■) or without (□) cholera toxin in the presence of various concentrations of NaCl, washed and then assayed for adenylate cyclase activity (see sections 2.1 and 2.2). The values quoted for adenylate cyclase activities are the means of duplicate determinations which agreed to within $\pm 10\%$. Similar results were obtained when the experiment was repeated.

adenylate cyclase activity
(pmol cAMP produced/min/
mg membrane protein)

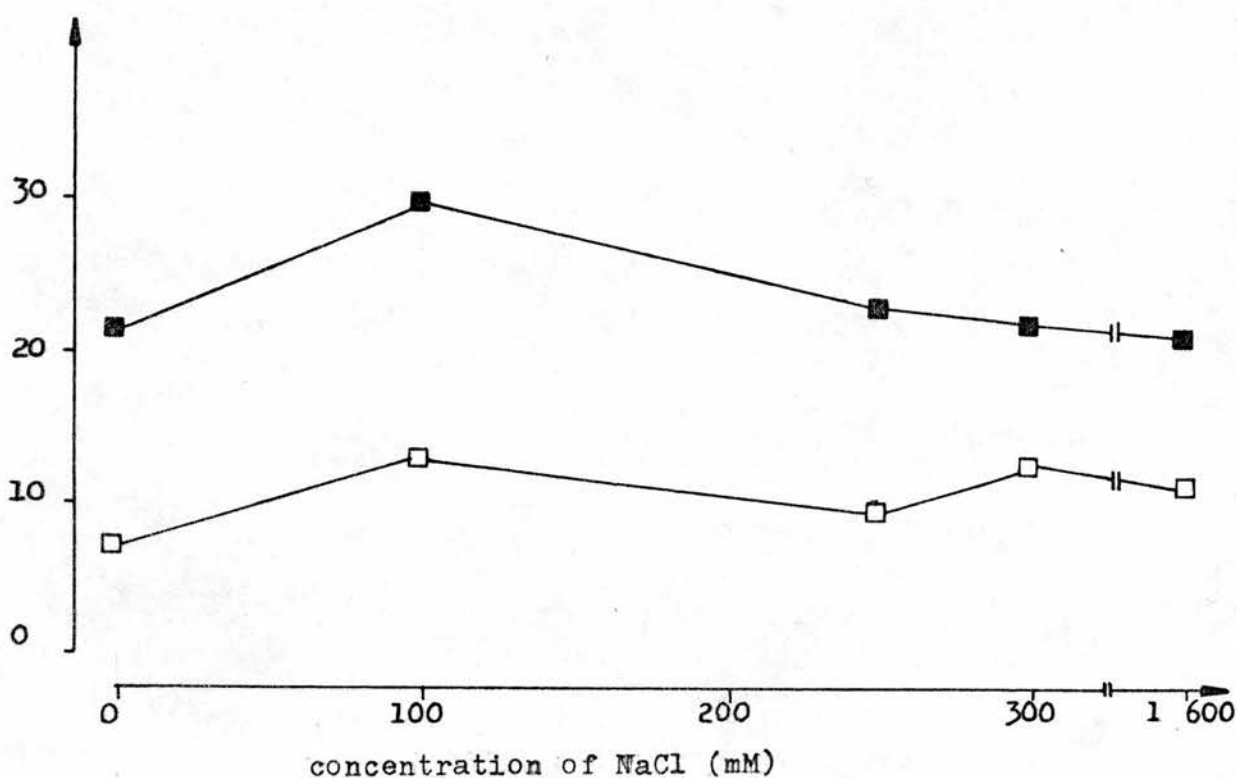
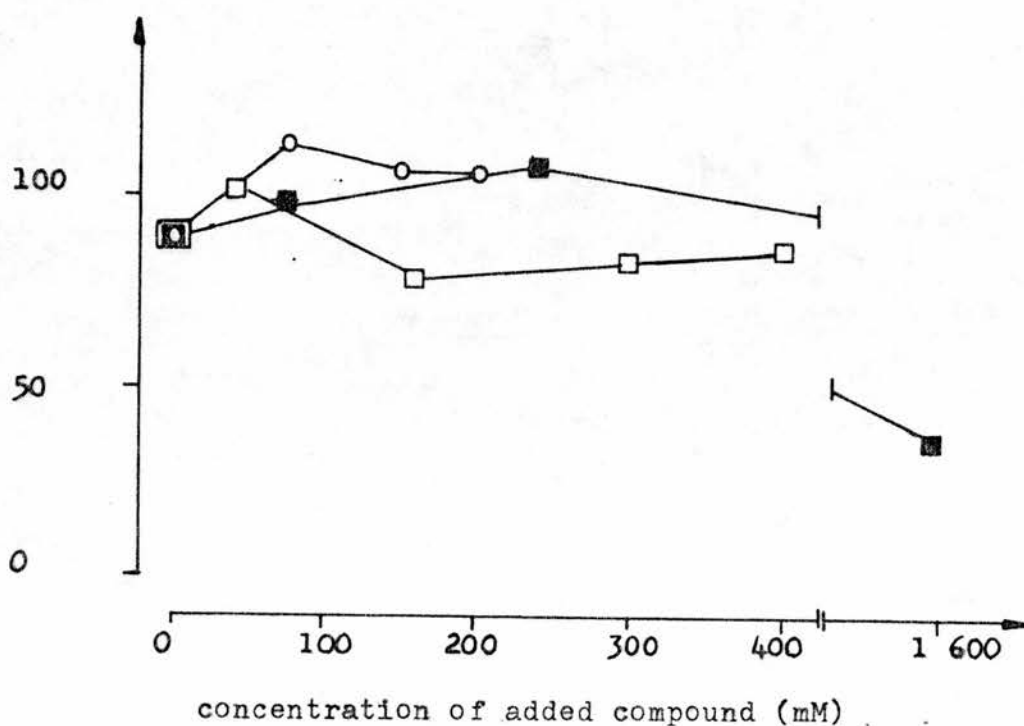


Fig. 2.7

The effects of various compounds on the stimulation of
adenylate cyclase by fluoride ions

Rat liver membranes were incubated with 20mM NaF in the presence of various other compounds, washed and then assayed for adenylylase activity. The additional compounds were L-arginine methyl ester (○), nicotinamide (□), or NaCl (■). Details of the experimental techniques are given in sections 2.1 and 2.2. The values quoted for adenylylase activities are the means of duplicate determinations which agreed to within $\pm 10\%$. Similar results were obtained when the experiment was repeated. The effects of the same compounds on the stimulation of adenylylase by cholera toxin and on the basal activity of the enzyme were also measured (see figs 2.6, 4.5 and 4.6).

adenylate cyclase activity
(pmol cAMP produced/min/
mg membrane protein)



The hydrolysis-resistant analogue of GTP, p(NH)ppG stimulated adenylate cyclase in rat liver membranes. 10 μ M p(NH)ppG caused maximal activation of the enzyme (fig. 2.8). No exogenous agent was required to stimulate the exchange of guanyl nucleotides so that p(NH)ppG activated adenylate cyclase. This result is very similar to that of Rodbell et al. (1975). Membranes were often pre-incubated with 1mM p(NH)ppG then assayed for adenylate cyclase activity to test for functional regulatory protein in the adenylate cyclase complex (see section 1.3.2).

2.3 Scintillation counting

The scintillation fluid used in this work was 700cm³ toluene containing 0.15g 1,4-di- $\sqrt{2}$ -(4-methyl-5-phenyloxazoly)-benzene and 5.5g 2,5-diphenyloxazole, made up to one litre with Triton X-100. Samples were usually counted on a Searle Mk III Liquid Scintillation System. This machine calculates dpm by the samples-channel-ratio method (see Peng, 1977). Alternatively samples were counted on a Packard 460C Liquid Scintillation System. This machine calculates dpm by the external-standard method (see Peng, 1977).

Many experiments involved dual-labelling with ³H and ³²P. In each case the cross-over from one channel into the other was determined by one of the following methods.

- (a) A series of standards containing only one of the labels was set up for each isotope. The mean relative cross-over was calculated from the apparent counts in each standard.
- (b) The samples were counted again after several months when the activity of ³²P was negligible. The ³H levels were not significantly changed from the first count.
- (c) Cross-over from the ³H channel into the ³²P channel was negligible. The flow in the reverse direction was estimated by counting the samples again after one half-life of ³²P



Fig. 2.8

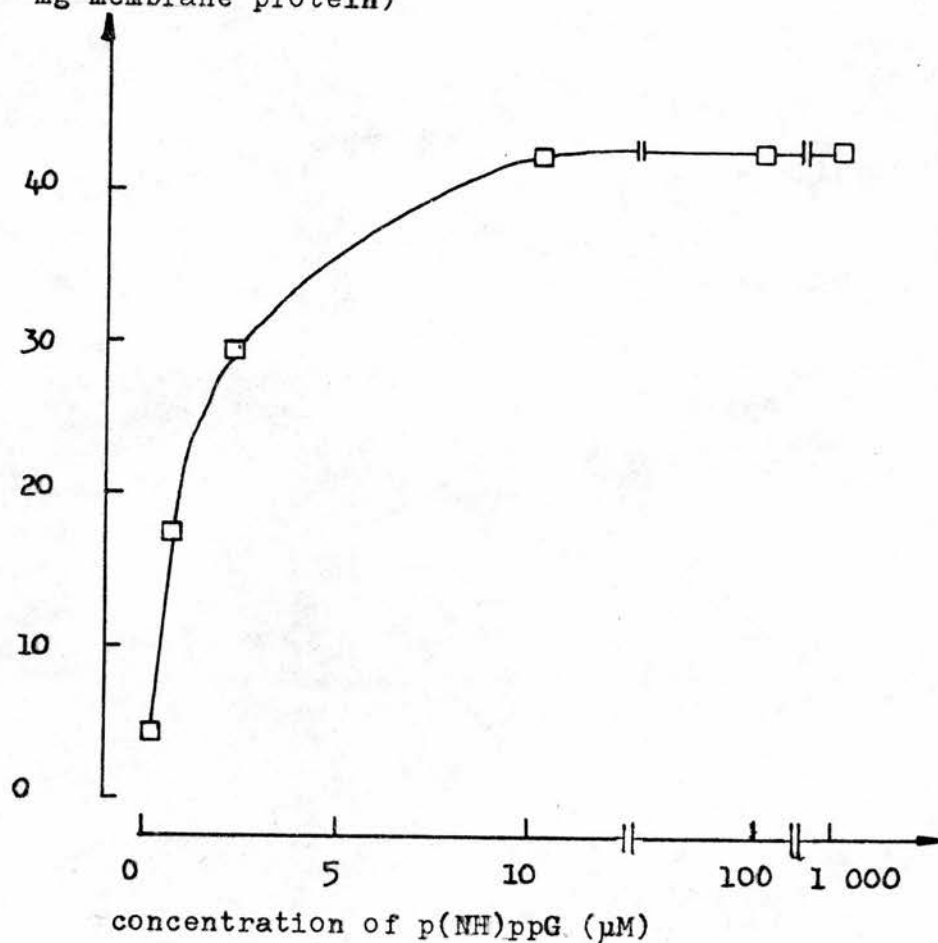
Activation of adenylate cyclase by p(NH)ppG

Rat liver membranes were incubated with various concentrations of p(NH)ppG, washed and then assayed for adenylate cyclase activity (see sections 2.1 and 2.2). The values quoted for adenylate cyclase activities are the means of duplicate determinations which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated.

adenylate cyclase activity

(pmol cAMP produced/min/

mg membrane protein)



(i.e. 14 days). After this time the ^{32}P counts in both channels were halved.

2.4 Determination of protein concentration

Protein was measured using the technique of Bradford (1976), which is based upon the change in E_{595} of Coomassie Brilliant Blue when it binds to protein. The assays were calibrated using BSA, taking its extinction coefficient at 280nm as $0.667\text{mg}^{-1}\text{cm}^3\text{cm}^{-1}$ (Reynolds et al., 1967).

2.5 Preparation of radioactive NAD^+

The technique used to make and purify radioactive NAD^+ was that of Cassel and Pfeuffer (1978). NAD^+ was produced by reacting radioactive ATP with NMN in the presence of NAD^+ -pyrophosphorylase (Boehringer Corporation (London) Ltd, Ealing, London) (fig. 2.9). The NAD^+ was purified by chromatography on Dowex 1-X8 (200-400 mesh) in the formate form, using 0.1M formic acid as the elution solvent. The resulting solution of NAD^+ was concentrated by freeze-drying. The compound was resuspended at about $3\text{mCi}/\text{cm}^3$ in ethanol:water (1:1) and stored at -20°C . These conditions reduced self-decomposition due to radioactive decay (Evans, 1976). The quantity and purity of the NAD^+ was assayed using chromatography on DEAE-paper (Tait and van Heyningen, 1978). After chromatography, the paper was dried, cut-up then added to scintillation fluid for counting as described in section 2.3. NAD^+ was typically obtained at 90-95% radiochemical purity and 25-35% yield relative to initial ATP. These values are similar to those reported by other workers (Cassel and Pfeuffer, 1978; Johnson et al., 1978).

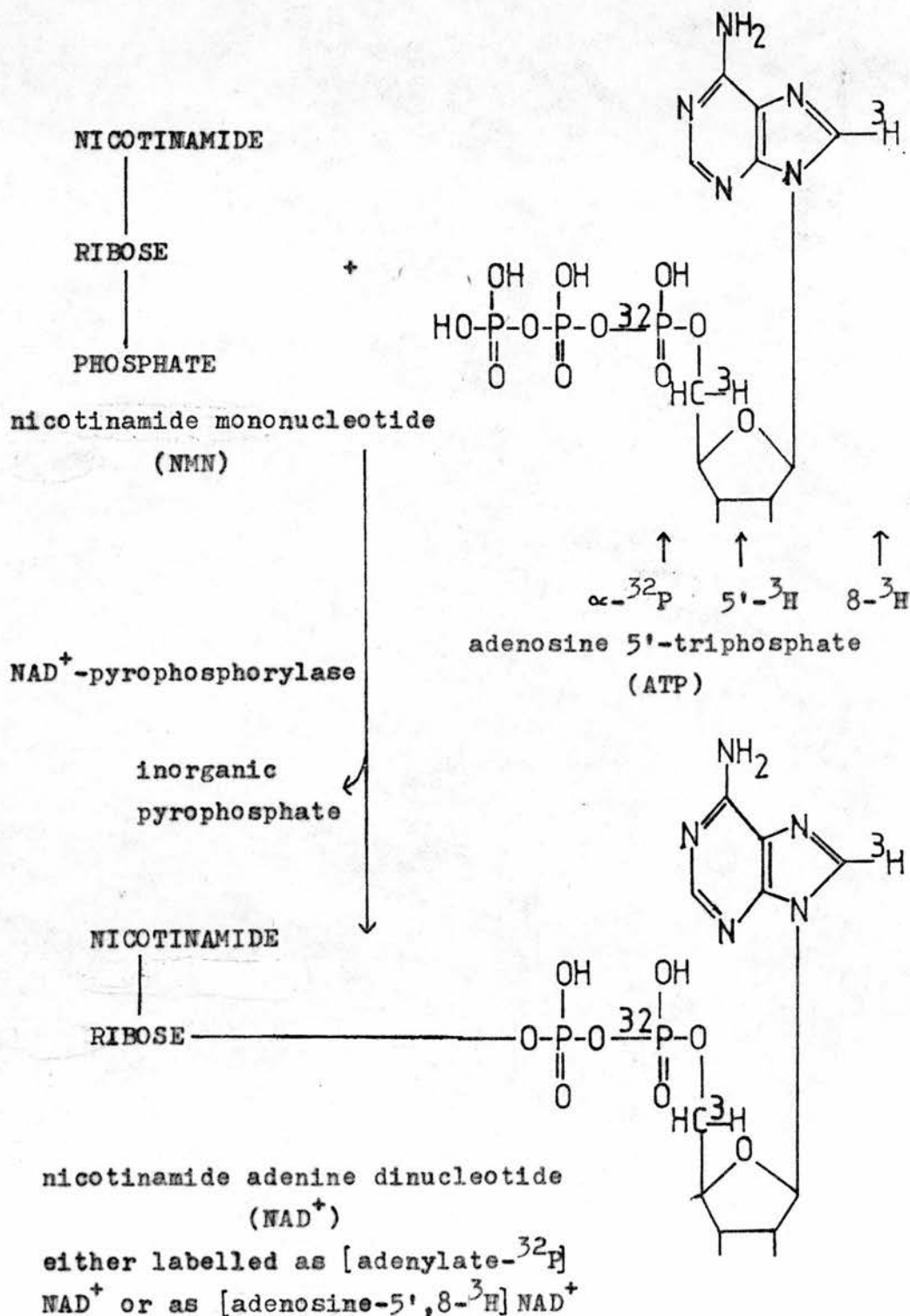
2.6 Labelling of proteins in rat liver membranes using cholera toxin and radioactive NAD^+

Membranes were prepared then incubated with preactivated cholera toxin as described in section 2.1. The only modification was that NAD^+ was used at about $10\mu\text{M}$ and $20\text{-}50\text{Ci}/\text{mmol}$. The NAD^+ was

Fig. 2.9

The reaction between NMN and ATP to produce NAD^+

This reaction was used to produce radioactive NAD^+ from labelled ATP (see section 2.5). ATP was labelled with either ^3H or ^{32}P .



either labelled as $\text{[adenylate-}^{32}\text{P]}$ or $\text{[adenosine-5',8-}^3\text{H]}$ (see fig. 2.9). All of the experiments using $\text{[}^3\text{H]NAD}^+$ were performed using material which was made from $\text{[}^3\text{H]ATP}$ (see section 2.5). $\text{[}^{32}\text{P]NAD}^+$ which was prepared from $\text{[}^{32}\text{P]ATP}$ was only used in preliminary studies and no results from this work appear in this thesis. $\text{[}^{32}\text{P]NAD}^+$ became available commercially and this material was used for most of my experiments. After incubation with radioactive NAD^+ , membranes were washed three times in perfusion buffer. Centrifugation was at room temperature in capped plastic tubes in a Beckman Microfuge at about 10 000 g_{max} for 2min and at a fixed angle. Solutions containing radiolabelled proteins were treated with benzamidine to 1mM and PMSF to 0.1mM to inhibit proteolysis (see section 2.7).

The conditions for this incubation were the same as those which produced maximal activation of adenylate cyclase by cholera toxin (figs. 2.4 and 4.3). Labelling of membrane proteins by cholera toxin also reached completion (fig. 4.4).

The NAD^+ used for studying ADP-ribosylation was in water: ethanol (1:1, v/v). This resulted in a final concentration of about 5% (v/v) ethanol in the incubation mixture. 10% (v/v) ethanol had no effect on the activation of adenylate cyclase by cholera toxin (table 2.5) therefore it is unlikely that 5% ethanol produced artefactual results in ADP-ribosylation studies.

2.7 Protective agents used in buffers

The following chemicals were added to buffers when the preservation of enzyme activity was not required. Sodium azide (0.02% w/v) was used to prevent the growth of bacteria (Dawson *et al.*, 1979). 1mM benzamidine and 0.1mM phenylmethanesulphonyl fluoride (PMSF) to inhibit serine proteases (Jeffcoate and White, 1974; Fahrney and Gold, 1963). PMSF was stored in propan-2-ol as it breaks down rapidly in aqueous solution. Despite this, PMSF is an effective inhibitor of serine proteases as it irreversibly modifies the active site (Fahrney and Gold, 1963). A fresh aliquot of the reagent is needed at each step in the experiments.

Table 2.5

The effects of ethanol on the activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin and ethanol, washed, then assayed for adenylate cyclase activity (see sections 2.1 and 2.2). The values which are quoted are the means of duplicate determinations which agreed to within $\pm 5\%$.

<u>final concentration</u> <u>of ethanol (v/v)</u>	<u>treatment with</u> <u>cholera toxin</u>	<u>adenylate cyclase activity</u> <u>(pmol cAMP produced/min/ mg protein)</u>
0	-	22.8
0	+	45.1
10%	-	22.5
10%	+	40.8

2.8 Polyacrylamide-gel electrophoresis (PAGE)

2.8.1 General procedure

PAGE was routinely conducted under denaturing conditions (i.e. in the presence of 0.1% w/v SDS). The gel and buffer system was based upon that of Laemmli (1970) with the addition of 2mM EDTA to chelate metal ions which may interfere with the polymerisation of acrylamide and cause aggregation of proteins (Douglas and Butow, 1976). Polyacrylamide ($M_r > 5 \times 10^6$) was added to 0.5% (w/v) in the separating gel to increase its mechanical strength. Slab gels (about 15cm x 15cm x 1.3mm) were electrophoresed in vertical tanks as described by Studier (1973). Treatment with 2-mercaptoethanol and SDS breaks proteins down into their constituent polypeptides. The procedure for staining the polypeptides after electrophoresis is given in table 2.6.

Staining solution was made by dissolving Coomassie Blue in methanol, then filtering through Whatman No. 1 paper before the addition of acetic acid. All of the processes in the staining procedure were carried out at 55°C.

Gels were dried, using a Hoeffer slab gel dryer, onto Whatman 3mm paper under vacuum and with heating. The dryer was used according to the manufacturer's instructions.

The mobility of polypeptides in SDS-PAGE is linearly related to the $\log M_r$ (Weber et al., 1972). The M_r of a polypeptide can be determined by comparison of mobility with molecules of known M_r . When gels were used to determine M_r , proteins of known M_r were run on the same gel as the samples. Markers of M_r were selected from the list in table 2.7, and data was fitted to a straight line by the nonparametric method of Nimmo and Atkins (1979). A typical calibration graph and further details of the experimental technique are given in fig. 2.10.

Table 2.6

Staining procedure for SDS-polyacrylamide-gels

	<u>process</u>		
	<u>fixing</u>	<u>staining</u>	<u>destaining</u>
<u>time</u>	15min	15min	until back-ground is clear
<u>composition of reagent</u>			
methanol (v/v)	20%	55%	5%
acetic acid (v/v)	10%	10%	7.5%
Coomassie Brilliant Blue R		0.25% (w/v)	

The general procedure for SDS-PAGE is given in section 2.8.1.

Table 2.7

M_r-standards for SDS-PAGE

<u>protein</u>	<u>source</u>	<u>M_r of poly-peptides</u> <u>(x10⁻³)</u>	<u>reference</u>
ribonuclease	bovine pancreas	13.7	Weber & Osborn (1969)
myoglobin	horse muscle	17.2	Weber & Osborn (1969)
immunoglobulin G	bovine	23.5 and 50	Weber & Osborn (1969)
alcohol dehydro- genase	yeast	37	Weber & Osborn (1969)
aldolase	rabbit muscle	37	Lee & Horecker (1974)
creatine kinase	rabbit muscle	40	Weber & Osborn (1969)
ovalbumin	egg	43	Weber & Osborn (1969)
pyruvate kinase	rabbit muscle	57	Weber & Osborn (1969)
serum albumin	bovine	66.2	Reed <u>et al.</u> , (1975)
phosphorylase <u>b</u>	rabbit muscle	94	Cohen <u>et al.</u> , (1971)

Phosphorylase b was a gift from Professor P. Cohen (Department of Biochemistry, University of Dundee).

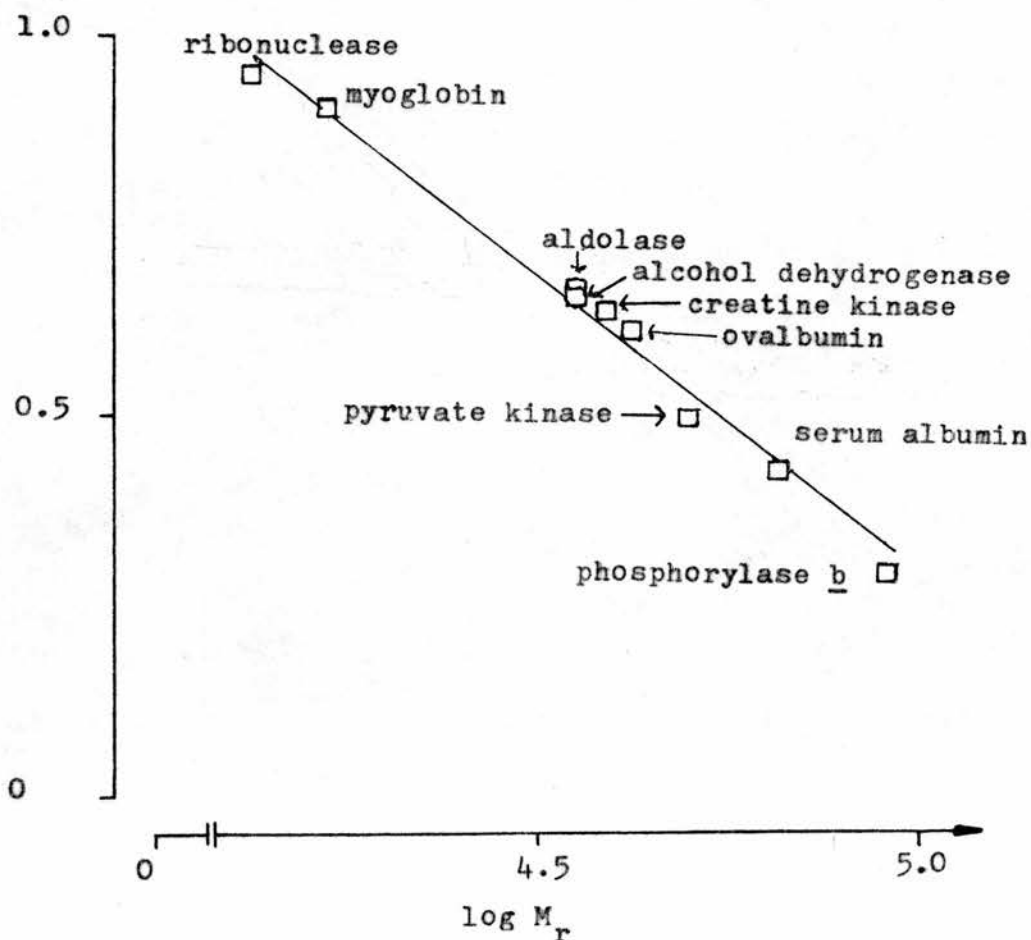
The use of M_r-standards for SDS-PAGE is described in section 2.8.1.

Fig. 2.10

Calibration graph for $\log M_r$ against relative mobility on SDS-PAGE

M_r -standards from table 2.7 were subjected to SDS-PAGE in a separating gel containing a final concentration of 8%(w/v) acrylamide (see section 2.8). The mobility of each polypeptide was measured relative to that of bromophenol blue (which was added to each sample as a marker-dye, see Weber and Osborn, 1969). The relationship between the mobilities and the $\log M_r$ s of the polypeptides could be represented as a straight line (see below). For each gel-system this is only true for polypeptides that are within certain limits of size (Weber and Osborn, 1969). To accurately measure the M_r of a polypeptide of unknown size, its relative mobility must fall on the straight part of the calibration graph. At least six M_r -standards (see table 2.7) were used to calibrate each gel.

relative mobility



This technique enabled the determination of M_r of polypeptides to within $\pm 5\%$. Proteins were detected with a higher resolution when the gel was stained rather than when the distribution of radioactivity was determined, as decay by ^{32}P has a high energy producing broad bands on autoradiography. The resolution in determining the position of radioactive bands in sliced gels (see section 2.8.2) is limited by the thickness of the slices. When radiolabelling was the only method of detection the error reached $\pm 10\%$.

2.8.2 Digestion of polyacrylamide-gels for scintillation counting

Polyacrylamide quenches radiation by tritium, therefore gels were sliced then digested with the strong base NCS (Nuclear Chicago Solubiliser, Amersham/Searle Corporation, Arlington Heights, Illinois, USA) before scintillation counting. NCS undergoes chemiluminescent reactions with the gel and scintillation fluid therefore it was acidified before addition of the latter, and incubated before counting (Peng, 1977).

After electrophoresis gels were not fixed, but were immediately cut into 1mm slices which were placed in small scintillation vials then covered with 0.2cm^3 90% (v/v) NCS. The preparations were sealed with plastic stoppers and incubated at $60-70^\circ\text{C}$ for 3h then stored overnight at room temperature. Next $7\mu\text{l}$ concentrated HCl was added, the samples were mixed, then 4cm^3 scintillation fluid was added. Samples were counted after storage for 4-7 days in the dark at room temperature.

2.9 Autoradiography

2.9.1 General procedure

Gels were dried down (see section 2.8.1) then exposed to Agfa Curix RP1 X-ray film at -70°C . (Low temperature decreases the background on the film, see Rogers (1979).) Films were pre-flashed by a single discharge from a Braun Hobby 17B flash gun through a Wratten No. 22 filter held 1m from the film.

This increased the sensitivity of the film to radiation (Rogers, 1979). Ideally the films were exposed between Dupont Cronex Lightning Plus intensifying screens (Dupont (U.K.) Ltd, Stevenage, Herts.). These exposure holders contained sheets of calcium tungstate which fluoresce when exposed to high-energy radiation (Laskey & Mills, 1977; Swanstrom & Shank, 1978). The procedure for developing X-ray film after exposure is given in table 2.8.

The solutions were used five times. After fixing the film was extensively washed with tap-water then hung up to dry at room temperature.

2.9.2 Quantification of amount of radiolabel exposed to X-ray film

The film was pre-flashed and always exposed for the same period of time (48h) between intensifying screens. Autoradiographs were scanned for E_{600} then the amount of radiolabel was estimated from the areas under the peaks (assuming a local background level to be the bottom of the peak) (see fig. 2.11). There was a linear relationship between peak area and amount of ^{32}P up to 2 000cpm (fig. 2.12). A calibration curve was constructed for each batch of experiments using ^{32}P . Each gel was exposed to film from the same box. This technique enabled estimation of incorporation of radioactivity to within $\pm 10\%$ in the range of 300-2 000cpm/peak.

2.9.3 Measurement of ADP-ribosylation by cholera toxin

The specific activity of the $[^{32}\text{P}]\text{NAD}^+$ used for ADP-ribosylation studies was known. This enabled calculation of the amount of incorporation of material into polypeptides. This was a measurement of ADP-ribosylation, not of ADP-ribosyltransferase activity, as the amounts of cholera toxin and NAD^+ , and incubation times used were sufficient to ensure that toxin-specific reactions reached completion (figs. 2.4 and 4.4). This technique did not specifically detect ADP-ribosylation,

Table 2.8

Procedure for developing X-ray film

	<u>process</u>		
	<u>developing</u>	<u>stopping developing</u>	<u>fixing</u>
<u>time (min)</u>	5	5	5
<u>composition of reagent</u>	17% (v/v) Agfa G150	5% (v/v) acetic acid	19.5% (v/v) Agfa G334, 2.4% (v/v) Agfa aditan hardner*

*Agfa G334 was diluted before addition of the hardner.

The procedure for autoradiography using X-ray film is given in section 2.9.1.

Fig. 2.11

Method of measuring peak areas on extinction scans of autoradiographs

The peak areas were used to estimate the amount of radioactivity that was associated with each band on autoradiographs (see section 2.9.2).

extinction at 600nm

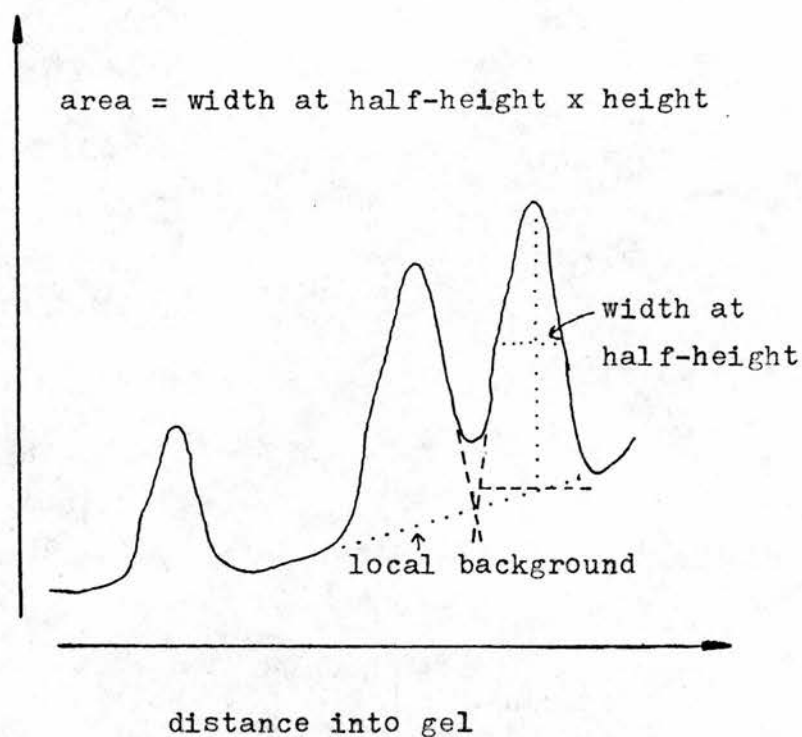
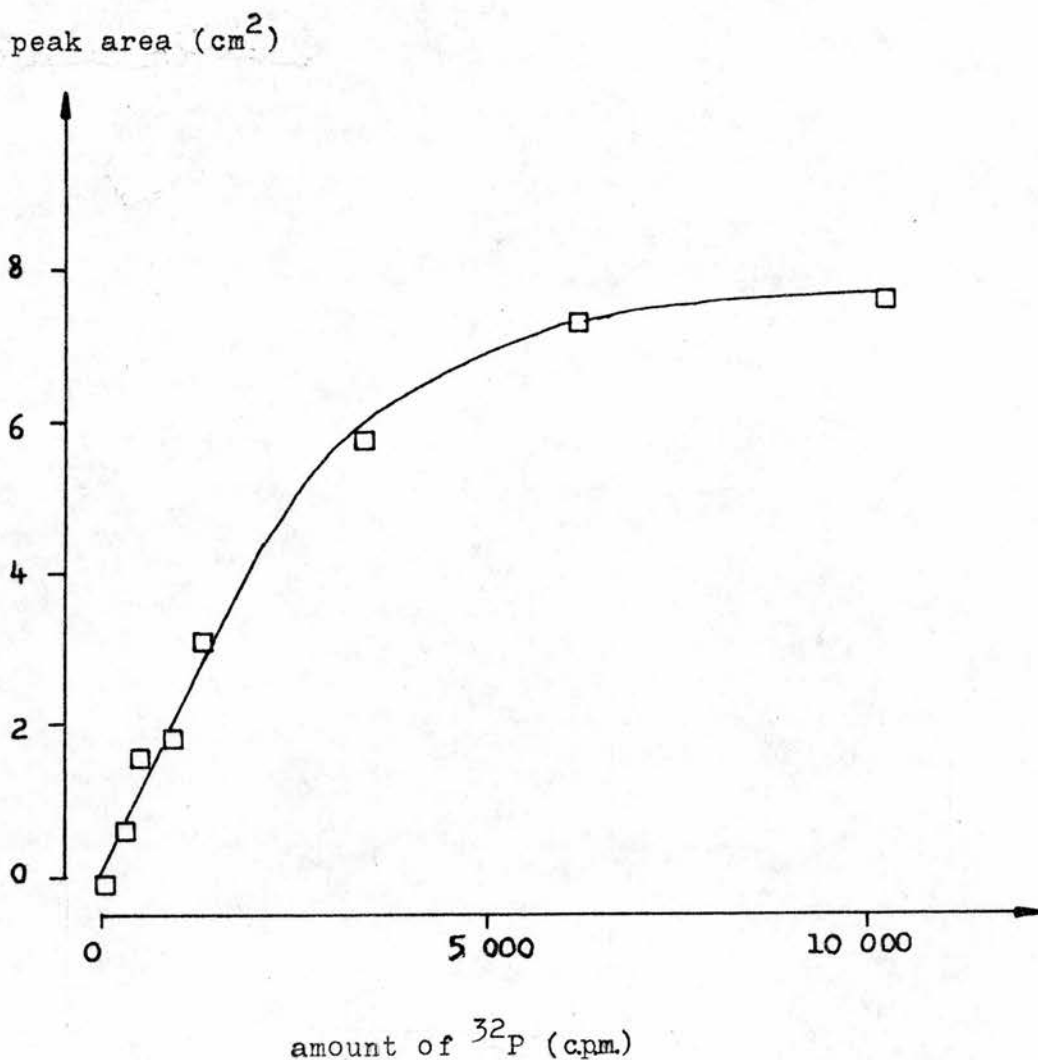


Fig. 2.12

Relationship between peak area on extinction scans of autoradiographs and amount of radiolabel in each band

Known amounts of ^{32}P were dried onto Whatman 3mm paper which was then exposed to X-ray film. The autoradiograph was developed, scanned to measure the extent of blackening and then the area under each peak on the scan was estimated (see section 2.9.2). Each point is the mean of duplicate measurements which agreed to within $\pm 10\%$.



but it did measure the incorporation of radioactivity from the ADP-ribose moiety of NAD^+ into polypeptides. The amount of radiolabel incorporated, relative to the NAD^+ in the incubation was very low. It is possible that radioactive metabolites or contaminants had donated the labelling group. The results presented later (section 4.2.3) and in the introduction (section 1.3.3) make this very unlikely.

2.10 Gel-permeation chromatography

Two gel-permeation columns were poured and used according to the manufacturers' instructions. A column (1m x 1.3cm) of Sephacryl S-200 (Pharmacia, Hounslow, Middlesex, U.K.) was eluted at about $10\text{cm}^3/\text{h}$. The Ultrogel AcA34 column (LKB Instruments Ltd, South Croydon, Surrey, U.K.) (1m x 2.5cm) was eluted at about $18\text{cm}^3/\text{h}$. PMSF (a protease inhibitor, see section 2.7), blue dextran and 5-N dinitrophenyl lysine were added to the sample, which was made dense by the addition of sucrose to about 10% (w/v). Blue dextran was added to determine the elution volume (V_o) for molecules which were excluded from the gel, and 5-N dinitrophenyl lysine eluted with molecules which were able to diffuse readily into the beads (at a volume that is referred to as V_s). This enabled determination of the distribution coefficients of the proteins which were fractionated on the columns (see below). The proteins were eluted at 4°C with 10mM HEPES, 5mM MgCl_2 , 100mM NaCl, 0.7% (w/v) Lubrol PX, 1mM benzamidine, 0.02% (w/v) sodium azide adjusted to pH7.4 with NaOH.

The columns were calibrated with a selection of proteins so that the distribution coefficient (K_D) could be related to the apparent M_r (table 2.9, and figs. 2.13 and 2.14) (see Fischer, 1980). K_D is given by:

$$K_D = \frac{V - V_o}{V_s - V_o}$$

Table 2.9

Proteins used to calibrate gel-permeation columns

<u>protein</u>	<u>source</u>	<u>M_r (x10⁻³)</u>	<u>reference</u>
ovalbumin	egg	43	Weber & Osborn (1969)
haemoglobin	human	64	Darnall & Klotz (1975)
serum albumin	bovine	66	Reed <u>et al.</u> (1972)
dimeric serum albumin*	bovine	132	Reed <u>et al.</u> (1972).
immunoglobulin G	bovine	147	Weber & Osborn (1969)
alcohol dehydrogenase	yeast	150	Darnall & Klotz (1975)
creatine kinase	rabbit muscle	160	Weber & Osborn (1969)
catalase	bovine liver	232	Darnall & Klotz (1975)

*Serum albumin dimerised spontaneously.

The use of proteins to calibrate gel-permeation columns is described in section 2.10.

Fig. 2.13

Calibration graph for the Sephacryl S-200 column

Marker proteins from table 2.9 were used to calibrate the column (1m x 1.3cm) of Sephacryl (see section 2.10). Proteins were chromatographed individually and then again in different combinations. This enabled identification of which protein was associated with which peak of E_{280} . Then all of the proteins were applied as a single sample. Each time the column was eluted at about $10\text{cm}^3/\text{h}$ and fractions containing about 1.2cm^3 were collected. E_{280} was measured in alternate fractions to identify the elution positions of the proteins. K_D values were calculated as described in section 2.10. Similar results were obtained when the experiment was repeated a further twice.

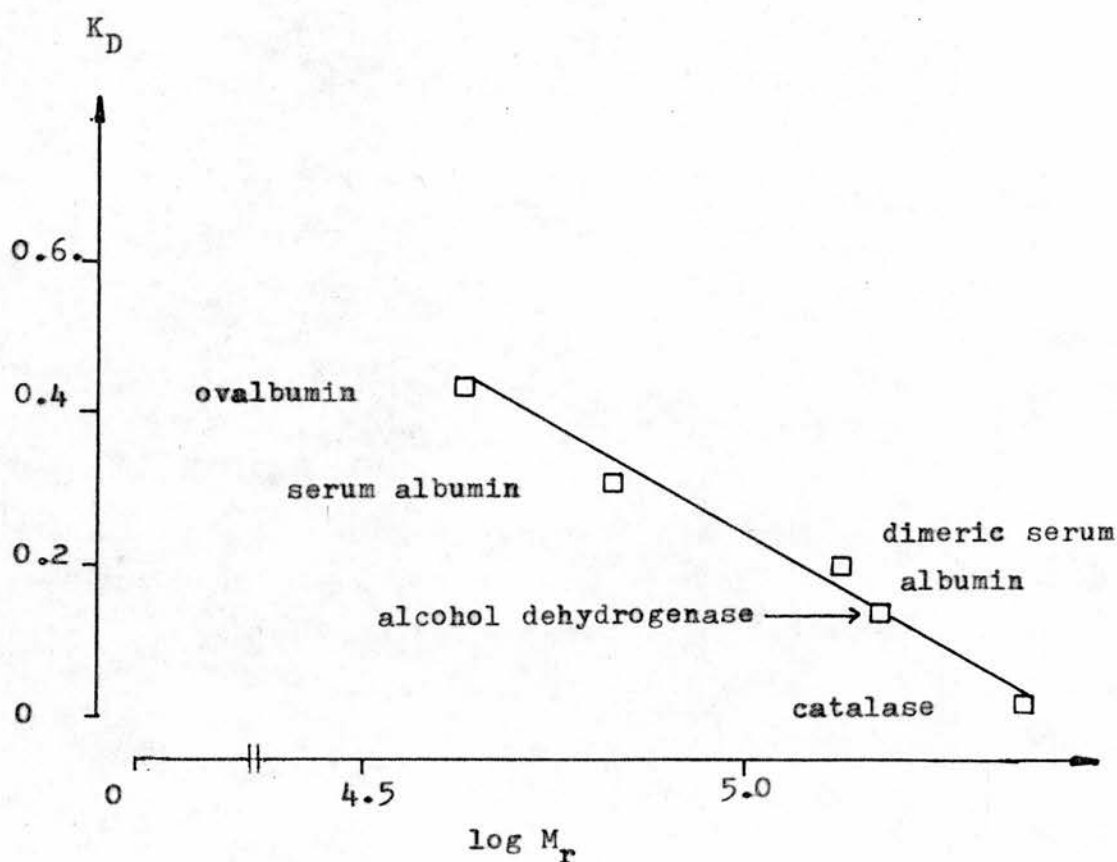
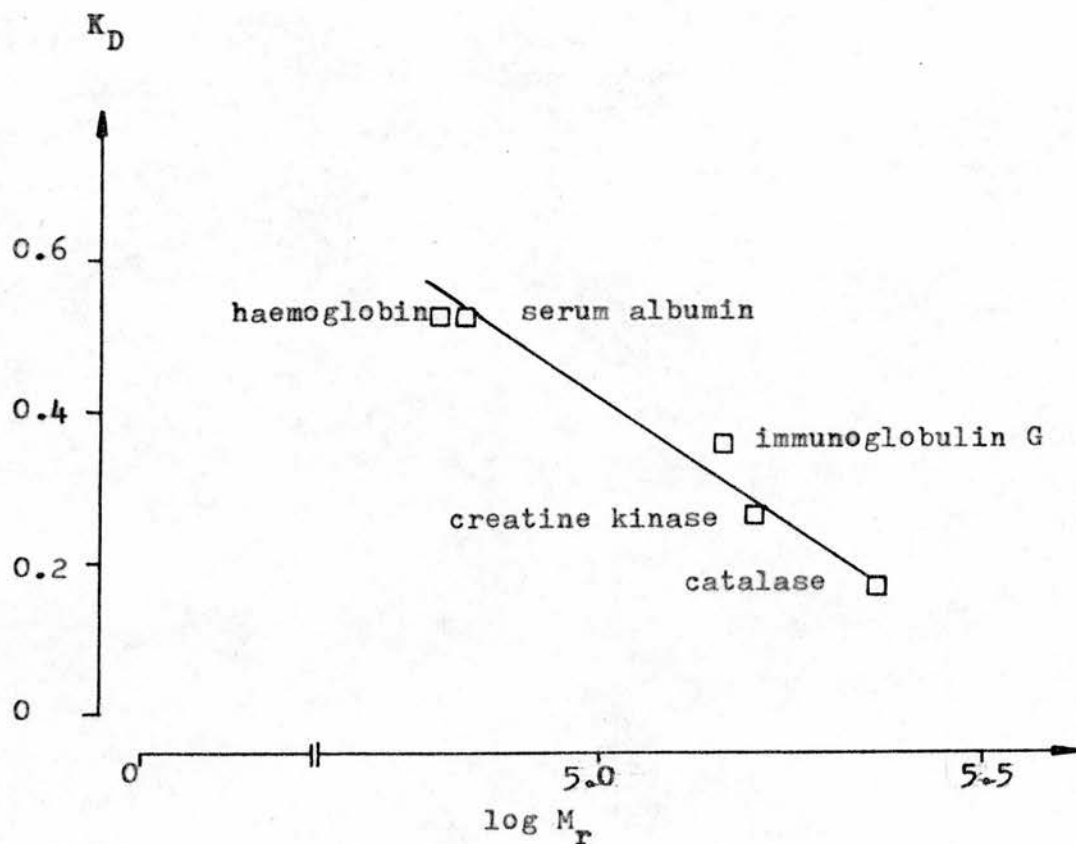


Fig. 2.14

Calibration graph for the Ultrogel AcA34 column

Marker proteins from table 2.9 were used to calibrate the column (1m x 2.5cm) of Ultrogel (see section 2.10). Proteins were chromatographed individually and then again in different combinations. This enabled identification of which protein was associated with which peak of E_{280} . Then all of the proteins were applied as a single sample. Each time the column was eluted at about $18\text{cm}^3/\text{h}$ and fractions containing about 5cm^3 were collected. E_{280} was measured in alternate fractions to identify the elution positions of the proteins. K_D values were calculated as described in section 2.10. Similar results were obtained when the experiment was repeated.



where V_e = elution volume for a particular protein. Both V_o and V_s are defined above. The lines of best-fit were calculated by the nonparametric method of Nimmo and Atkins (1979).

There was a linear relationship between K_D and $\log M_r$ of proteins (figs 2.13 and 2.14). The Sephacryl column fractionated molecules of lower apparent M_r than those resolved by the Ultrogel column.

Estimates of M_r made on the basis of K_D are very tentative for the following reasons.

- (a) Proteins may aggregate.
- (b) Proteins could be solubilized in complexes containing other proteins, lipids or detergent.
- (c) Some proteins may have unusual shapes.
- (d) Proteins may interact with the bed material of the column, or with other molecules in the sample or in the elution buffer.

Gel-permeation chromatography was used to estimate M_r to within $\pm 10\%$.

3. THE HYDROPHOBICITY OF CHOLERA TOXIN IN RELATION TO ITS ACTION ACROSS PLASMA MEMBRANES

3.1 EXPERIMENTAL

3.1.1 Prediction of hydrophobicity on the basis of amino-acid composition and sequence

There are several ways to predict the hydrophobicity of a protein. The techniques which were used in the current work are explained below.

- (a) The average hydrophobicity, \bar{H}_ϕ , of a protein is given by:

$$\bar{H}_\phi = \sum_i \Delta G_{ti} \chi_i$$

where ΔG_t is the free-energy of transfer of amino-acids from ethanol to water, and χ_i is the mole-fraction of the amino-acid in the protein (Bigelow, 1967). The values of ΔG_t which were used in this work are given in table 3.1.

- (b) The ratio of frequency of occurrence is given by:

$$R = \frac{k \chi_k}{j \chi_j}$$

where χ is the mole-fraction of a given amino-acid. For R_3 , k = arginine, lysine, histidine, glutamate, glutamine, aspartate and asparagine, and j = isoleucine, tyrosine, phenylalanine, leucine, valine and methionine (Barrantes, 1975).

- (c) A discriminant function, z , was found to be a useful combination of \bar{H}_ϕ and R_3 as it distinguished well between proteins of different hydrophobicities (Raftery et al.,

Table 3.1

ΔG_p values and relative hydrophobicity values used to predict hydrophobicity of proteins

These values were taken from Tanford (1962), Nozaki and Tanford (1971) and Segrest and Feldman (1974). Methods for predicting the hydrophobicity of a protein are given in section 3.1.1.

<u>amino-acid</u>	<u>ΔG_p (kcal/mole)</u>	<u>relative hydrophobicity</u>
tryptophan	3.40	6.5
isoleucine	2.97	5.0
tyrosine	2.30	4.5
phenylalanine	2.50	5.0
leucine	1.80	3.5
valine	1.50	3.0
methionine	1.30	2.5
cysteine	1.00	0.0
alanine	0.50	1.0
glycine	0.00	0.0
histidine	0.50	1.0
proline	2.60	1.5
serine	-0.30	-0.5
threonine	0.40	0.5
asparagine	-0.01	-1.5
glutamine	-0.10	-1.0
aspartate	0.54	} not hydrophobic
glutamate	0.55	
lysine	1.50	
arginine	0.75	

1975). z is defined as:

$$z = 0.60\overline{H}_\phi - 0.345R_3$$

when \overline{H}_ϕ is expressed in kcal/mole.

- (d) The hydrophobicity index, HI, of a peptide is the mean relative hydrophobicity per amino-acid residue. By definition, only peptides containing at least ten consecutive hydrophobic residues (i.e. amino-acids that have a relative hydrophobicity value, see table 3.1) have a HI. The relative hydrophobicity of each residue is assigned loosely on the basis of ΔG_t (see table 3.1 section 3.2.1 and Segrest and Feldman, 1974).

3.1.2 Detection of hydrophobic surfaces on proteins using charge-shift electrophoresis

Charge-shift electrophoresis was introduced by Helenius and Simons (1977). The binding of the non-ionic detergent Triton X-100 (Koch-Light Ltd., Colnbrook, Bucks) to hydrophobic areas on the surface of the protein is observed by determining the charge on the protein in detergent mixtures containing Triton and anionic detergent (sodium deoxycholate, DOC), or Triton and cationic detergent (cetyltrimethylammonium bromide, CTAB) (DOC and CTAB were from Sigma, see appendix I). High ionic-strength is used so that ionic detergents do not bind to charged groups on the surface of the protein. Molecules which bind Triton electrophorese in opposite directions in each mixture of detergents.

Before electrophoresis, samples (at 1-2mg protein/cm³) were incubated at 4°C overnight with charge-shift buffer at four times its concentration in the gel. Electrophoresis was at room temperature in 1%(w/v) agarose (agarose [ME] from Miles, see appendix I) on microscope slides (3cm³ agarose per slide) with paper wicks (Whatman No. 1). The gel buffer was 0.05M

glycine, 0.1M NaCl adjusted to pH9.0 with NaOH, containing:

- (a) no detergent, or
- (b) Triton X-100 at 5g/l, or
- (c) Triton X-100 at 5g/l and DOC at 2.5g/l, or
- (d) Triton X-100 at 5g/l and CTAB at 0.5g/l.

The same buffer was used in the electrophoresis tank as in the gel except for the 'Triton-only' experiments where there was no detergent in the tank buffer. Gels were pre-electrophoresed for 15min at 9Vcm^{-1} and then the samples (10-20 μg protein) were applied and electrophoresed for 5h at 9Vcm^{-1} . The gels were then treated as described in table 3.2.

The ability of the charge-shift system to detect hydrophobic areas on the surfaces of proteins was tested using molecules of known hydrophobicity (tables 3.3 and 3.6).

3.1.3 Testing the activities of cholera toxin and its subunits after pretreatment for charge-shift electrophoresis

The proteins (at $1\text{-}2\text{mg cm}^{-3}$) were incubated with charge-shift buffers (at four times their concentrations in the gels) overnight at 4°C (see section 3.1.2).

After this pretreatment cholera toxin and its A subunit were preactivated in the normal way (see section 2.1.4) and then assayed for the ability to stimulate adenylate cyclase in rat liver membranes (see sections 2.1 and 2.2).

The activity of cholera toxin and its B subunits was measured using their ability to bind to ganglioside GM1. 0.5mg ganglioside GM1 and 4.5mg cerobroside were dissolved in 1cm^3 chloroform:methanol(1:1,v/v). (The lipids were purified from

Table 3.2

Treatments for gels after charge-shift electrophoresis

The procedure for charge-shift electrophoresis is given in section 3.1.2.

<u>treatment</u>	<u>reagent</u>	<u>conditions</u>
fixing of protein and removal of detergent	20% (v/v) methanol, 10% (v/v) acetic acid	soaked overnight at room temperature.
drying of gel		gel covered with wet filter paper (Whatman No. 1) and then allowed to dry at room tempera- ture. The paper was moistened and then removed
staining of protein	0.7g/l Coomassie Brilliant Blue R, 45% (v/v) methanol, 9 % (v/v) acetic acid*	left at room tempera- ture for 90min
destaining of gel	7.5% (v/v) acetic acid, 5% (v/v) methanol	room temperature, the reagent was changed as necessary

*Coomassie Blue was dissolved in methanol and then filtered through paper (Whatman No. 1) before adding acetic acid.

Table 3.3

Sources of protein samples for charge-shift electrophoresis

These samples were used to test if the charge-shift system detected hydrophobic areas on the surfaces of proteins (see section 3.1.2 and table 3.5).

<u>protein</u>	<u>source</u>	<u>are there hydrophobic areas on the surface of the protein?</u>
diphtheria toxin	a gift from Wellcome Research Laboratories, Beckenham, Kent	no (Boquet, 1979)
apoprotein B from human low D.L.	} gifts from Dr I. F. Craig, Department of Biochemistry, University of Edinburgh	yes (Morrisett <u>et al.</u> , 1975)
apoprotein A1 from human high D.L.		
cytochrome b ₅₆₁ (from bovine chromaffin granules)	a gift from Mr J. G. Pryde, Department of Biochemistry, University of Edinburgh	yes (Apps <u>et al.</u> , 1981)

D.L. = density lipoprotein.

Bovine serum albumin, immunoglobulin G (bovine) and ovalbumin were obtained from Sigma (see appendix I). None of these proteins have hydrophobic surfaces (see Helenius and Simons, 1977).

bovine brain by N. Gascoyne, see King et al. (1976).) 50 μ l of this solution was dispensed into each of several plastic centrifuge tubes and then dried using N₂. This produced complexes of ganglioside and cerebroside (van Heyningen, 1974) which were scraped to the bottom of the tubes. 10 μ l water and 23 μ l protein in detergent (30-50 μ g protein) was added and then the preparations were shaken in a water-bath for 1h at 37°C. Controls contained cerebroside as the only lipid. The tubes were then centrifuged at about 10 000g_{max} for 5min at room temperature. This pelleted the lipids (which were insoluble) together with any protein that had bound to them. The proteins in the supernatants were identified by SDS-PAGE (see section 2.8).

3.2 RESULTS

3.2.1 Predicted hydrophobicities of cholera toxin and its components

Predictions of hydrophobicity are unreliable as the theory behind them is over-simplified. The fact that the ranges of hydrophobicities for different classes of proteins overlap considerably shows the poor resolution of the technique (see table 3.4). Predictions that are based upon amino-acid composition estimate the hydrophobicity averaged over the whole of the molecule. The effects of the three-dimensional structure of the proteins and of the local environments of the side chains of amino-acids are ignored in calculations of hydrophobicity. Peptide bonding does change the charges on the side chains of amino-acids when they become incorporated into proteins.

Measurements of ΔG_t would probably have been more useful if α -ester derivatives of amino-acids were used. ΔG_t is a poor measure of hydrophobicity in several cases. A hydrophobic amino-acid has a positive ΔG_t , but aspartate and glutamate had more positive values than their amides. This is probably because the acids associate in solution to produce aggregations with polar interiors and non-polar exteriors. The amides do not do this as readily as the acids. Glutamine has a more hydrophilic ΔG_t value than asparagine, but glutamine possesses an extra methylene group and therefore must be more hydrophobic than asparagine. This is because asparagine aggregates in aqueous solution in a similar way to aspartate, but to a lesser extent. Asparagine forms these associations more readily than glutamine (see Nozaki and Tanford, 1974). Lysine and arginine are two of the most polar amino-acids, but they have relatively large ΔG_t values so that they act as hydrophobic amino-acids in predictions.

Estimates of hydrophobicity which are based upon the primary structure of a protein are more reliable than those based upon the amino-acid composition. This is because, in the predictions which were based upon sequence, the hydrophobicity scale was

Table 3.4

Hydrophobicities of cholera toxin and its subunits predicted from their amino-acid compositions

<u>protein</u>	<u>estimate of hydrophobicity</u>		
	<u>\bar{H}_p (kcal/mol)</u>	<u>\bar{R}_3</u>	<u>\bar{z}</u>
non-membrane proteins (n = 205)	0.90 - 1.09	0.84 - 1.68	-0.10 - 0.33
external membrane proteins (n = 24)	0.91 - 1.06	1.02 - 1.72	-0.04 - 0.28
internal membrane proteins (n = 24)	1.10 - 1.29	0.41 - 0.77	0.41 - 0.63
<u>cholera toxin</u>			
A1 polypeptide	0.94 - 1.07	1.29	0.12 - 0.20
A2 polypeptide	0.89	1.69	-0.05
A subunit	0.93 - 1.03	1.37	0.08 - 0.15
B subunit	1.05	1.34	0.16 - 0.17
A2./B/5 complex	1.03 - 1.04	1.39	0.14
complete toxin	1.01 - 1.05	1.35	0.14 - 0.16

The ranges of estimates of hydrophobicity for standard proteins were calculated by Barrantes (1975) and Raftery et al. (1975) (see section 3.1.1). The range quoted represents mean \pm standard deviation, and n indicates the number of proteins which were studied.

Values for cholera toxin and its components were determined on the basis of the data of Lai (1980) and Duffy et al. (1981b) (see section 3.1.1).

based on ΔG_t and then modified to correct for the anomalies which are described above (see table 3.1), also this technique detects local hydrophobic areas rather than the average hydrophobicity of the whole protein.

The structural data on cholera toxin is incomplete. The hydrophilic aspartate and glutamate are often not differentiated from their relatively hydrophobic amides. Tryptophan, which is the most hydrophobic amino-acid, is not usually determined in amino-acid analyses and neither is it considered in calculations of R_3 (see section 3.1.1 and Barrantes, 1975). Much of the amino-acid sequence of the A1 polypeptide from cholera toxin is not known. The complete primary structure is published for the A2 polypeptide and the B subunit of cholera toxin (Duffy et al., 1981b; Nakashima et al., 1976).

All of the methods for predicting hydrophobicity from the amino-acid compositions suggested that cholera toxin and its components are hydrophilic (table 3.4). A range of values is quoted for cholera toxin and its constituents because aspartate and glutamate were not always discriminated from their amides.

There were several hydrophobic sequences of amino-acids in cholera toxin and in the very similar A subunit of E.coli labile toxin (see section 1.4 and table 3.5). (Part of the gene for the A subunit of E.coli labile toxin has been sequenced by Spicer et al. (1981).) But the hydrophobicity indices of these sequences were comparable with those found by Segrest and Feldman (1974) for the interiors of proteins, rather than those for hydrophobic proteins. A peptide which is sufficiently hydrophobic, and long enough, to extend across a membrane would have a hydrophobicity index of > 2.75 and contain at least ten amino-acid residues (Segrest and Feldman, 1974). The parts of cholera toxin that have been sequenced contain no such peptides. Insufficient data were available to align the sequenced fragments from the A1 polypeptide, therefore it was impossible to estimate the hydrophobicity of the

Table 3.5

Hydrophobic sequences in cholera toxin

<u>source of sequence</u>	<u>identity of sequence</u>	<u>hydrophobicity index</u>
A1 polypeptide	fragment CnI: (Lai <u>et al.</u> , 1979)	
	phenylalanine 1 - tyrosine 27	1.31
	glx 18 - tyrosine 27	1.85
	N-terminal fragment: (Mendez <u>et al.</u> , 1975)	
	glx 1 - proline 15	0.77
	proline 3 - proline 12	1.25
B subunit	tyrosine 12 - asparagine 21	1.20
	threonine 92 - asparagine 103	1.85

glx = glutamate or glutamine. asx = aspartate or asparagine.

Hydrophobicity indices were calculated by the method of Segrest and Feldman (1974) (see section 3.1.1). All asx and glx residues were taken to be amides as this gave the maximum possible hydrophobicities. All of the hydrophobic sequences in cholera toxin are given above. Data on the primary structure of cholera toxin was published by Mendez et al. (1975), Kurosky et al. (1976), Nakashima et al. (1976), Lai et al. (1979) and Duffy et al. (1981b).

unsequenced portions from the amino-acid composition.

3.2.2 Detection of hydrophobic surfaces on proteins using charge-shift electrophoresis

The charge-shift system was shown to be a valid technique using several proteins of known hydrophobicity (table 3.6).

The preparations of cholera toxin and its subunits appeared to be pure on SDS-PAGE (fig. 3.1). All three proteins had no charge-shift in the different mixtures of detergents showing that they had no hydrophobic areas on their surfaces (fig. 3.2). A minor (probably insignificant) component in the preparation of B subunit appeared to be hydrophobic. As the preparation of B subunit was pure, this material must have been produced by a change in conformation from the predominant form, or it may be artefactual.

3.2.3 The activities of cholera toxin and its subunits after pretreatment for charge-shift electrophoresis

After pretreatment with the detergents that were used in the charge-shift experiments cholera toxin and A subunit both retained the ability to activate adenylate cyclase in rat liver membranes (table 3.7). As the proteins catalyze the activation process (rather than acting stoichiometrically) this assay might not detect that a large proportion of the protein had been denatured. But the amounts of protein that were used were not large excesses over the minimum amounts that were required for maximum activation of adenylate cyclase. ($10\mu\text{g}/\text{cm}^3$ cholera toxin or $15\mu\text{g}/\text{cm}^3$ A subunit was used, whereas $7\mu\text{g}/\text{cm}^3$ or $13\mu\text{g}/\text{cm}^3$ respectively was required to produce maximal activation of adenylate cyclase (figs. 2.4 and 2.5).) Therefore a large proportion of cholera toxin and A subunit probably retained their active conformations during charge-shift electrophoresis. As the concentrations of detergents and ions were lower in the incubations with the membranes than in

Table 3.6

Migration of proteins on charge-shift electrophoresis

	<u>detergent mixture</u>				were hydro- phobic areas detected on the protein surface?
	no detergent	Triton alone	Triton + DOC	Triton + CTAB	
bovine serum albumin	+	+	+	+	no
ovalbumin	+	+	+	+	no
diphtheria toxin	+	+	+	+*	no
immunoglobulin G	0	0	0	0	no
apoprotein B from human low density lipoprotein	+	0	+	-	yes
apoprotein A1 from human high density lipoprotein	+	0	+	-	yes
cytochrome b ₅₆₁	+	0	+	not deter- mined	yes

+ indicates anodal migration, - indicates cathodal migration, 0 indicates no migration.

*Under these conditions diphtheria toxin split into two components as found by Boquet (1979). The same results were obtained when the experiment was repeated.

The procedure for charge-shift electrophoresis is given in section 3.1.2.

References for the hydrophobicities of these proteins are given in table 3.3.

Fig. 3.1

SDS-PAGE of cholera toxin and its subunits

Samples containing 30-50 μ g protein were electrophoresed in a separating gel which contained acrylamide to 15% (w/v) (see section 2.8). The proteins were purified by Mrs L. A. Rankine and Dr S. van Heyningen (see appendix 1). Lane 1, cholera toxin. Lane 2, A subunit. Lane 3, B subunit.

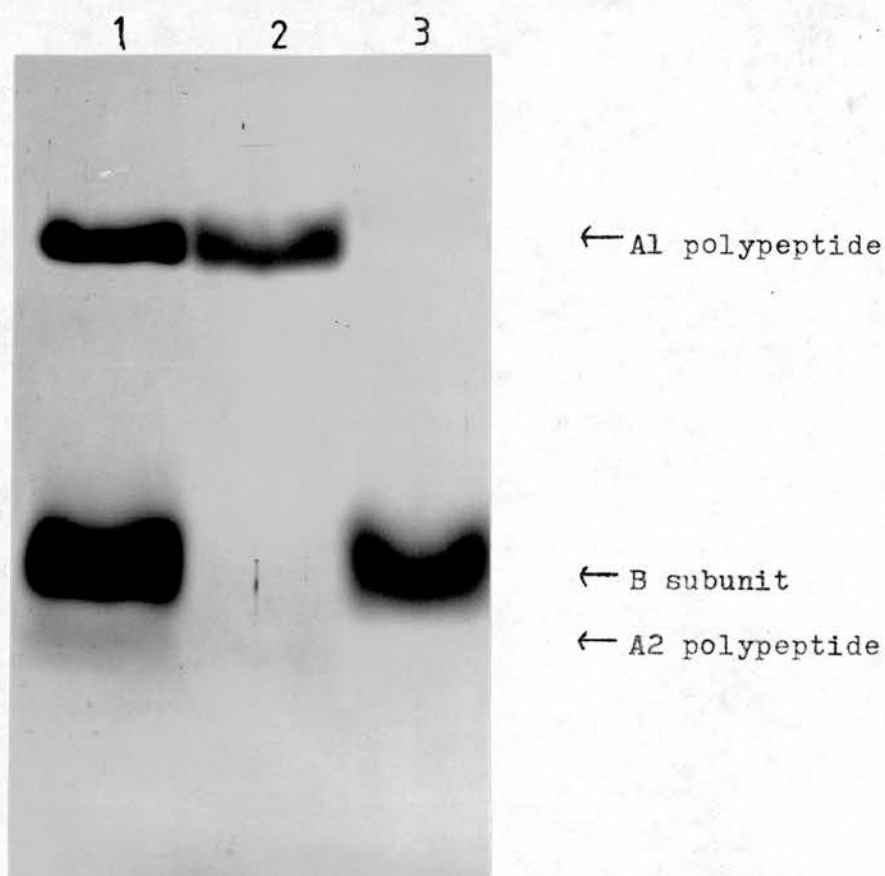


Fig. 3.2

Migration of cholera toxin and its subunits on charge-shift electrophoresis

Cholera toxin and its subunits were purified (see fig. 3.1) and then analysed by charge-shift electrophoresis (section 3.1.2).

The same results were obtained when the experiment was repeated.

1 = no detergent, 2 = Triton X-100 only, 3 = Triton X-100 plus DOC,
4 = Triton X-100 plus CTAB.

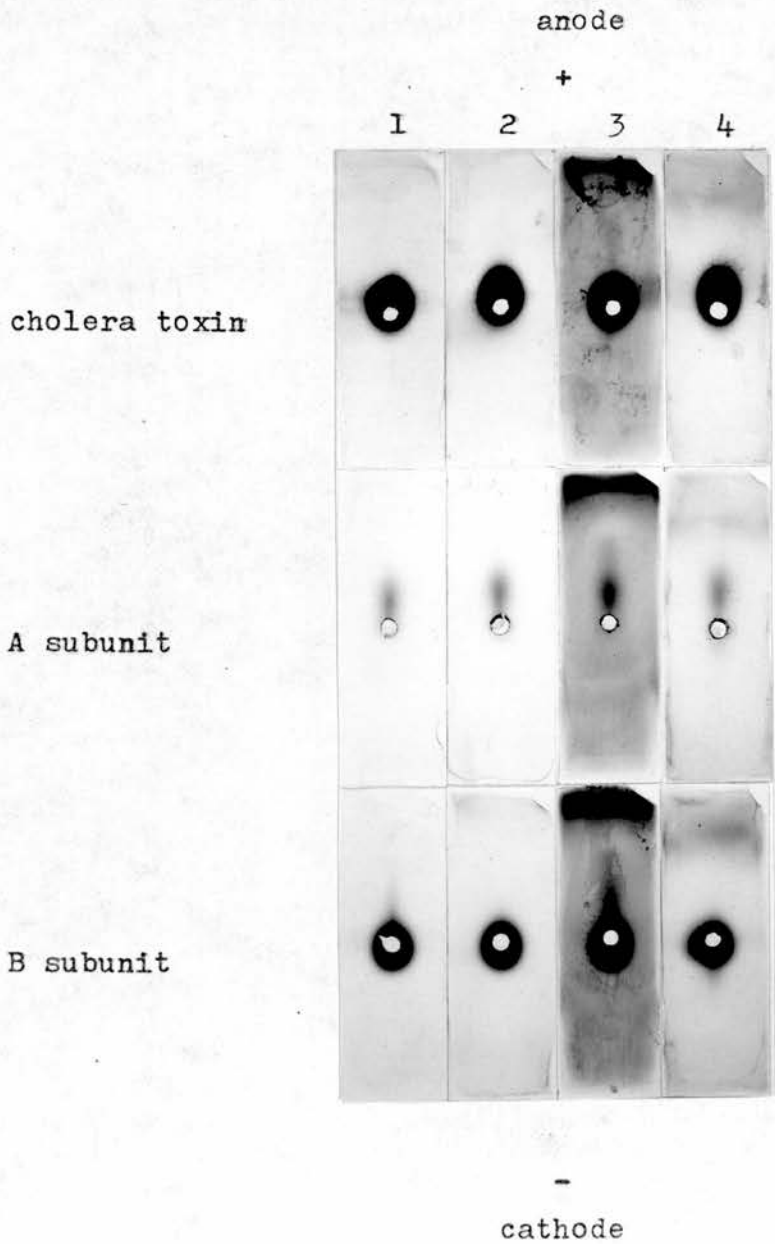


Table 3.7

Activation of adenylate cyclase by cholera toxin and its A subunit

The proteins were incubated as for charge-shift electrophoresis (see section 3.1.3) and then assayed for the ability to activate adenylate cyclase in rat liver membranes (see sections 2.1 and 2.2). The values are the means of duplicate determinations which agreed to within $\pm 10\%$. Similar results were obtained when the experiment was repeated.

adenylate cyclase activity (pmol cAMP produced/
mg membrane protein/min)

<u>detergents</u>	<u>basal</u>	<u>stimulated by A subunit</u>	<u>stimulated by cholera toxin</u>
none	11.5	25.6	30.4
Triton	13.2	29.1	23.2
Triton + DOC	10.4	22.6	20.5
Triton + CTAB	12.3	16.6	16.2

the charge-shift gels, this assumes that there was no reversible denaturation. Only one form of each protein could be seen on charge-shift electrophoresis, showing that any denatured protein had the same hydrophobicity as active protein (fig. 3.2).

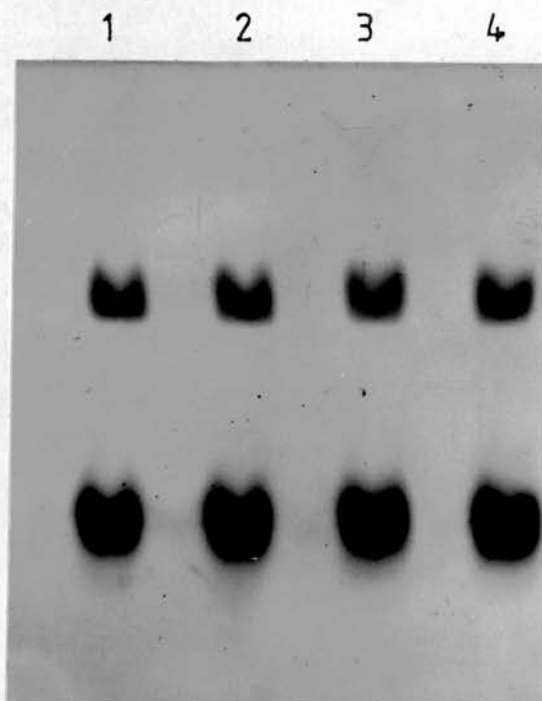
Cholera toxin bound to ganglioside GM1 which had been rendered insoluble by complexing it to cerebroside, but did not bind to cerebroside alone. Binding occurred even in the presence of detergents at twice their concentrations in the charge-shift gels (fig. 3.3). Identical results were obtained for the B subunit of cholera toxin. Therefore the proteins again appeared to be in active conformations during charge-shift electrophoresis.

Fig. 3.3

Binding of cholera toxin to complexes of ganglioside and cerebroside

Cholera toxin was preincubated with charge-shift detergents then mixed with complexes of ganglioside and cerebroside (left-hand lane of each pair) or with cerebroside alone for controls (right-hand lane of each pair). Unbound protein remained in the supernatant after centrifugation and was analysed by SDS-PAGE in a separating gel containing acrylamide to 15% (w/v) (see section 3.1.3). The same results were obtained when the experiment was repeated.

Pretreatment of cholera toxin: 1 = buffer, 2 = Triton X-100, 3 = Triton X-100 plus DOC and 4 = Triton X-100 plus CTAB.



3.3 DISCUSSION

The results of charge-shift electrophoresis show that the surfaces of cholera toxin and its subunits are all hydrophilic, and the theoretical calculations suggest that there are no masked hydrophobic regions in any part of the toxin.

Charge-shift electrophoresis measures the hydrophobicity of the surface of the protein in one particular conformation. The technique was especially useful in the current work as the proteins retained activity after the pretreatment for charge-shift electrophoresis. This is in agreement with the results of Helenius and Simons (1977) who found that, after charge-shift electrophoresis, proteins were still recognised by antibody which was raised to the same proteins in their active forms. This suggested that the proteins retained much of their three-dimensional structure. It is conceivable that the new conformations which are seen during the action of cholera toxin (see section 1.3.1) could be more hydrophobic than the active conformations which were studied here. But the results of the calculations (see section 3.2.1) show that it is unlikely that cholera toxin or its subunits could have hydrophobic surfaces, even after a conformation change. A conformation change in the A subunit (which usually has hydrophilic surfaces) could expose amphiphilic sequences. It has been suggested that the A2 polypeptide could act as a hydrophobic leader sequence for the entry of the A1 polypeptide into the plasma membrane (section 1.3.1.2). This would correlate with the observation that a fluorescent derivative of the A subunit showed a conformation change when cholera toxin bound to ganglioside GM1 (van Heyningen, 1982a). But the A2 polypeptide is not hydrophobic (section 3.2.1) and was not detected in the membrane by photoaffinity labelling (Wisniewski & Bramhall, 1979, see section 1.3.1.2).

The results of the current work do not support suggestions that the A1 chain partitions directly into the plasma membrane. But, as only very small amounts are needed inside the cell, such passage need not be a likely event (see section 1.3.1.2). Since the A1

polypeptide is hydrophilic, to enter the membrane it may need to interact with some integral membrane protein. There is evidence that the action of cholera toxin requires certain membrane proteins (Hagmann and Fishman, 1981, see section 1.3.1.2). The active part of the toxin may interact with several of the following proteins during its passage across the membrane.

- (a) It has been suggested that the receptor on the outside of cells for cholera toxin is a glycoprotein whose specificity is determined by the oligosaccharide moiety of ganglioside GM1. But this seems very unlikely (see section 1.3.1.1).
- (b) The active part of cholera toxin modifies the regulatory component of adenylate cyclase in the plasma membrane (see section 1.3.3.2). A polypeptide of $M_r = 45\ 000$ was ADP-ribosylated by cholera toxin in rat erythrocytes. This molecule was thought to be a component of the protein which regulates adenylate cyclase, and it appeared to be associated with the cytoskeleton (Sahyoun *et al.*, 1981b). Cholera toxin can catalyse the ADP-ribosylation of purified cytoskeletal proteins and may also modify them in plasma membranes (Kaslow *et al.*, 1981a). It could be that the components of adenylate cyclase and the cytoskeletal proteins interact with the active part of cholera toxin as they are less hydrophobic than most other parts of the interior of the plasma membrane.
- (c) The ADP-ribosyl transferase activity of cholera toxin which leads to the activation of adenylate cyclase is considerably enhanced by the reduction of the disulphide bond between the polypeptides of the A subunit (see section 1.3.3). This reduction process may be catalysed by protein disulphide reductase which is an enzyme in the plasma membrane (Moss *et al.*, 1980c).

Reduction of the disulphide bond in the A subunit of cholera toxin may also be required for the insertion of the A1 polypeptide into the lipid bilayer. Together with ganglioside GM1, photoactive probes were dissolved in the membranes of liposomes. The liposomes were then treated with cholera toxin. The A1 polypeptide only entered the membranes if the disulphide bond in the A subunit was reduced (Tomasi & Montecucco, 1981). (Proteolysis and reduction to release the A1 polypeptide from the A subunit were probably carried out by endogenous enzymes in the experiments using Newcastle Disease Virus which are described in section 1.3.1.2 (see Tomasi & Montecucco, 1981).) Protein disulphide reductase was found on the outer surface of external microvillous projections and on the inner surface of the plasma membranes of rat hepatocytes (Varandani et al., 1978). Cholera toxin may interact with this enzyme before the A1 polypeptide enters the membrane, but it seems unlikely that there could be a reducing agent outside the cell. Hydrophilic interactions with protein disulphide reductase may facilitate the transport of the active part of cholera toxin across the membrane.

The requirement for interaction with membrane proteins could explain the lag phase and dependence upon temperature during the action of cholera toxin on intact cells (see section 1.3.1). Hydrophilic derivatives of ganglioside GM1 were more potent than the ganglioside in inducing response to cholera toxin in intact cells (see section 1.3.1.2). This observation is consistent with the hypothesis that a hydrophilic molecule crosses the membrane. But this model does not explain the changes in permeability which were seen when artificial membranes were treated with cholera toxin (see section 1.3.1.2).

The results of charge-shift electrophoresis showed that the surface of the B subunit of cholera toxin is not hydrophobic. This observation is consistent with the fact that the B subunit was not labelled in photoaffinity experiments (Wisnieski and Bramhall, 1979; Wisnieski et al., 1979), suggesting this

part of cholera toxin does not enter the plasma membrane.

Cholera toxin may be taken up by endocytosis, but the A1 polypeptide would still have to cross the lysosomal membrane to activate adenylate cyclase. There is some indirect evidence that the protein does not cross this membrane, but rather reaches it as a result of lysosomal processing (see section 1.3.1.2). This work needs further investigation.

Cholera toxin was not significantly endocytosed into mouse thymus cells until 1-4h after they were treated with the toxin (Tsuru et al., 1982). The lag between the addition of cholera toxin and the activation of adenylate cyclase was not measured, but it is generally much shorter than 1-4h (see section 1.3.1), suggesting that, although endocytosis of cholera toxin may occur, it is not essential for the effect of the toxin on the cell.

The transmembrane action of cholera toxin probably involves a localised destabilisation of the membrane due to multivalent binding of ganglioside GM1. The B subunits would not enter the membrane, but a very high local concentration of A subunits would be induced. In the current work it was shown that cholera toxin and its components have no hydrophobic surfaces and no masked hydrophobic regions. Therefore part of cholera toxin probably interacts with polar molecules in the plasma membrane before exerting its effect inside the cell.

4. THE ACTIVATION OF ADENYLATE CYCLASE AND ADP-RIBOSYLATION BY CHOLERA TOXIN

4.1 EXPERIMENTAL

4.1.1. General procedure

Rat liver membranes were incubated with or without cholera toxin, washed, then adenylate cyclase activity or ADP-ribosylation was measured. The basic experimental techniques are described in section 2.

4.1.2 Attempted reversal of the activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin (section 2.1). The membranes were then washed three times with ice-cold 60mM MOPS. The pH of the medium used for the reversal incubation was varied. The pH of the MOPS used for washing was also varied (using NaOH), so that it was the same as that of the reversal medium. The composition of the incubation used in the reversal experiments was based upon that used by Gill and Meren (1978) (see table 4.1).

The mixture was incubated at 25°C for 30min, and the preparations were washed and resuspended at 10-15mg protein cm⁻³ in perfusion buffer (see section 2.1.2). Half of each sample was incubated with 20mM NaF at 25°C for 15min, washed and then assayed for adenylate cyclase activity to test the stability of the catalytic and regulatory components of the enzyme to the reversal procedure (see section 2.2.3). The remaining half of each sample received identical treatment except no NaF was added.

Table 4.1

Composition of the incubation medium used in reversal experiments

Experiments where the reversal of the action of cholera toxin was attempted are described in section 4.1.2.

rat liver membranes	10-15mg protein cm ⁻³
"preactivated" cholera toxin*	10-15µg cm ⁻³
nicotinamide	100mM
GTP	0.5mM
ATP	5mM
NADase (insoluble acetone powder from porcine liver)	0.5U cm ⁻³
MOPS	60mM
NaOH	to various pHs

*See section 2.1.4.

4.1.3 Covalent modification of proteins in rat liver membranes using phenylglyoxal

This procedure is based upon that of Franks et al. (1980).

Rat liver membranes were equilibrated with 1mM DTT, 12mM MOPS, containing NaOH to pH 7.4, by washing. Membranes were resuspended at about 5mg protein cm^{-3} in this buffer, then reacted with various concentrations of phenylglyoxal (Aldrich Chemical Co. Ltd, Gillingham, Dorset) in an equal volume of the same buffer. After incubation for 5min at 25°C, the membranes were washed four times in perfusion buffer to remove unreacted phenylglyoxal (see section 2.1.2). The membranes were resuspended at 20-25mg protein cm^{-3} in perfusion buffer, ready for incubation with cholera toxin (see section 2.1).

4.1.4 Solubilization of proteins from rat liver membranes

After incubation with or without cholera toxin, rat liver membranes were washed twice with perfusion buffer (see section 2.1.2) then solubilized in one of the following ways.

- (a) (Modified from van Heyningen, 1976 b). The membranes were resuspended at about 5mg protein cm^{-3} in perfusion buffer. The non-ionic detergent Lubrol PX was added to 0.5% (w/v) and then the mixture (about 1 cm^3) was ultrasonicated for 15s using a Rapidis 150 Ultrasonicator. The preparation was ultracentrifuged for 15min at 100 000 g_{max} and 2°C. The supernatant was added to an equal volume of Amberlite XAD-2 ion-exchange resin (BDH Chemicals Ltd, Poole, Dorset) which had been equilibrated with perfusion buffer. The mixture was incubated at 0°C for 3min with occasional shaking to allow excess detergent to bind to the resin. The extract was separated from the Amberlite using a Pasteur pipette.

(b) (Modified from Johnson et al., 1978). The membranes were resuspended at about $5\text{mg protein cm}^{-3}$ in perfusion buffer. Lubrol PX was added to 0.7%(w/v) and then the preparation was incubated on ice, with occasional shaking, for 1h. The mixture was centrifuged for 10min at 4°C , and about $10\,000g_{\text{max}}$. The clear supernatant contained the proteins that were solubilized from the membranes.

4.1.5 Complementation studies of adenylate cyclase

Rat liver membranes were incubated with or without cholera toxin and then washed twice and solubilized. The extracts were gradually added to a mixture of stored membranes and perfusion buffer. (Membranes had been stored at -70°C . Details are given in tables 4.9 and 4.10). The mixture was occasionally agitated gently during incubation on ice for 30min. $25\mu\text{l}$ samples were then assayed for adenylate cyclase activity at 15°C . Low temperature was used because the enzyme is very sensitive to heat after solubilization from rat liver (van Heyningen, 1976b).

4.1.6 Precipitation of solubilized proteins

Rat liver membranes were incubated with $\text{[adenylate-}^{32}\text{P]NAD}^{+}$ in the presence or absence of cholera toxin (section 2.6). After washing, proteins were solubilized from the membranes (section 4.1.4). Radiolabelled polypeptides had to be concentrated, and removed from excess detergent and buffer, before they could be analysed by SDS-PAGE and autoradiography (sections 2.8 and 2.9). This was achieved by precipitating the proteins as follows.

If necessary, BSA was added as carrier to increase the concentration of protein to 50ng cm^{-3} . The sample was treated with an equal volume of 10%(w/v) trichloroacetic acid and incubated, with occasional mixing, at 0°C for 30min. The mixture was centrifuged (at about $2\,500g_{\text{max}}$ and room

temperature for 5min) to pellet the precipitated protein, and then washed twice with ice-cold acetone. The protein was left to dry at room temperature for 2h, then resuspended in distilled water and sample buffer for SDS-PAGE (see section 2.8).

4.1.7 Two-dimensional electrophoresis of polypeptides

Proteins were electrophoresed along a pH gradient in a non-denaturing gel system until they reached their isoelectric points. This isoelectric focussing was the first dimension of the separation and was performed in glass tubes. The gel was removed from the tube and then placed on top of a SDS-polyacrylamide slab gel (see section 2.8). In this second dimension, polypeptides were separated according to size. The technique used was that described by Apps *et al.* (1980) except that 0.7%(w/v) Lubrol PX was used as the detergent in the sample buffer and in the first-dimension gel.

4.1.8 Fractionation of proteins solubilized from rat liver membranes

Rat liver membranes were incubated under various conditions (see legends in section 4.2), washed and then solubilized using method (b) in section 4.1.4. The proteins which were solubilized from the membranes were treated with inhibitors of proteolysis (see section 2.7) and then fractionated.

(a) Gel-permeation chromatography (see section 2.10)

Blue dextran and 5-N dinitrophenyl lysine were added to each sample before chromatography. This allowed accurate determination of K_D values. The density of the sample was increased by the addition of sucrose to 10%(w/v), this made loading onto the column easier.

(b) Ion-exchange chromatography

A column containing 0.2cm^3 Whatman DE-52 cation exchange

resin was poured according to the maker's instructions. The resin was poured into a Pasteur pipette which had been modified by removing most of the capillary to decrease the "dead-volume" below the plug of glass wool. Proteins were eluted in 10mM HEPES containing 5mM MgCl_2 , 0.7%(w/v) Lubrol PX, 1mM benzamidine and 0.02%(w/v) sodium azide, and the mixture was adjusted to pH7.4 with NaOH (the reasons for adding benzamidine and sodium azide are given in section 2.7). The concentration of NaCl was increased step-wise (4cm^3 per step) from 0-2M.

(c) Ammonium sulphate fractionation

The starting buffer for ion-exchange chromatography (i.e. that without NaCl) was saturated with ammonium sulphate and the pH was re-adjusted to 7.4 with NaOH. This buffer was added step-wise to the preparation of solubilized membrane proteins. After each addition, the mixture was incubated on ice for 30min with occasional shaking. Proteins were pelleted by centrifugation at 4°C , and $10\,000g_{\text{max}}$ for 20min.

4.2 RESULTS

4.2.1 Identification of the substrates of cholera toxin in rat liver membranes

Rat liver membranes were incubated with or without cholera toxin and radioactive NAD^+ (see section 2.6). The polypeptides were separated by SDS-PAGE and then the radiolabelled products were identified. The experiments were repeated many times and the results can be summarised as follows.

Several polypeptides were labelled even in the absence of cholera toxin. The predominant ones had M_r 's of about 110 000 and 20-25 000. The endogenously labelled molecule of $M_r \approx 110$ 000 is probably poly(ADP-ribose) polymerase (which was reported to be radiolabelled using NAD^+ , and to have a polypeptide of $M_r \approx 115$ 000 by Benjamin and Gill, 1980). The heterogeneous group of low M_r endogenously labelled polypeptides are probably components of histones. When membranes were incubated in the absence of cholera toxin, and with low concentrations of radioactive NAD^+ (about $5\mu\text{M}$), no labelling of polypeptides was detected (see fig. 4.15). But if membranes were incubated with $10\mu\text{M}$ NAD^+ , then endogenous radiolabelling was seen (figs 4.1 and 4.2). Cholera toxin transferred radioactivity from NAD^+ to polypeptides at both concentrations of the nucleotide suggesting that its affinity for the nucleotide was greater than that of the endogenous enzymes. The principal substrates of cholera toxin had approximate M_r 's of 42 000 and 47 000 (see figs 4.1 and 4.2). These receptors were shown to be polypeptides as they were digested by proteolytic enzymes (see section 5.2). A substrate of $M_r = 56$ 000 was sometimes apparent, but this polypeptide was also labelled endogenously (or comigrated with a polypeptide which was labelled endogenously) (fig. 4.1). The cholera toxin-specific increase in radiolabelling of the polypeptide of $M_r = 56$ 000 could be seen more clearly when membranes were incubated with [γ -adenosine-5', 8- ^3H] NAD^+ and gels were sliced and then counted

Fig. 4.1

Radiolabelling of polypeptides in rat liver membranes using
 γ -adenylate- ^{32}P /NAD $^{+}$

Rat liver membranes were incubated with γ -adenylate- ^{32}P /NAD $^{+}$ in the presence or absence of cholera toxin (section 2.6). Radiolabelled polypeptides were analysed by SDS-PAGE followed by autoradiography (sections 2.8 and 2.9). The concentration of acrylamide in the separating gel was 8% (w/v). Values of M_r were estimated as described in section 2.8. Myoglobin and the light chain from immunoglobulin G both had high mobilities and electrophoresed into the bottom part of the gel which is not included in this photograph. This experiment was repeated six times giving similar results. The minor bands that were seen after incubation with cholera toxin (arrowed) were not consistently detected (e.g. see fig. 4.15). About 0.5mg protein was loaded into each lane of the gel.

membranes incubated
without cholera toxin



membranes incubated
with cholera toxin



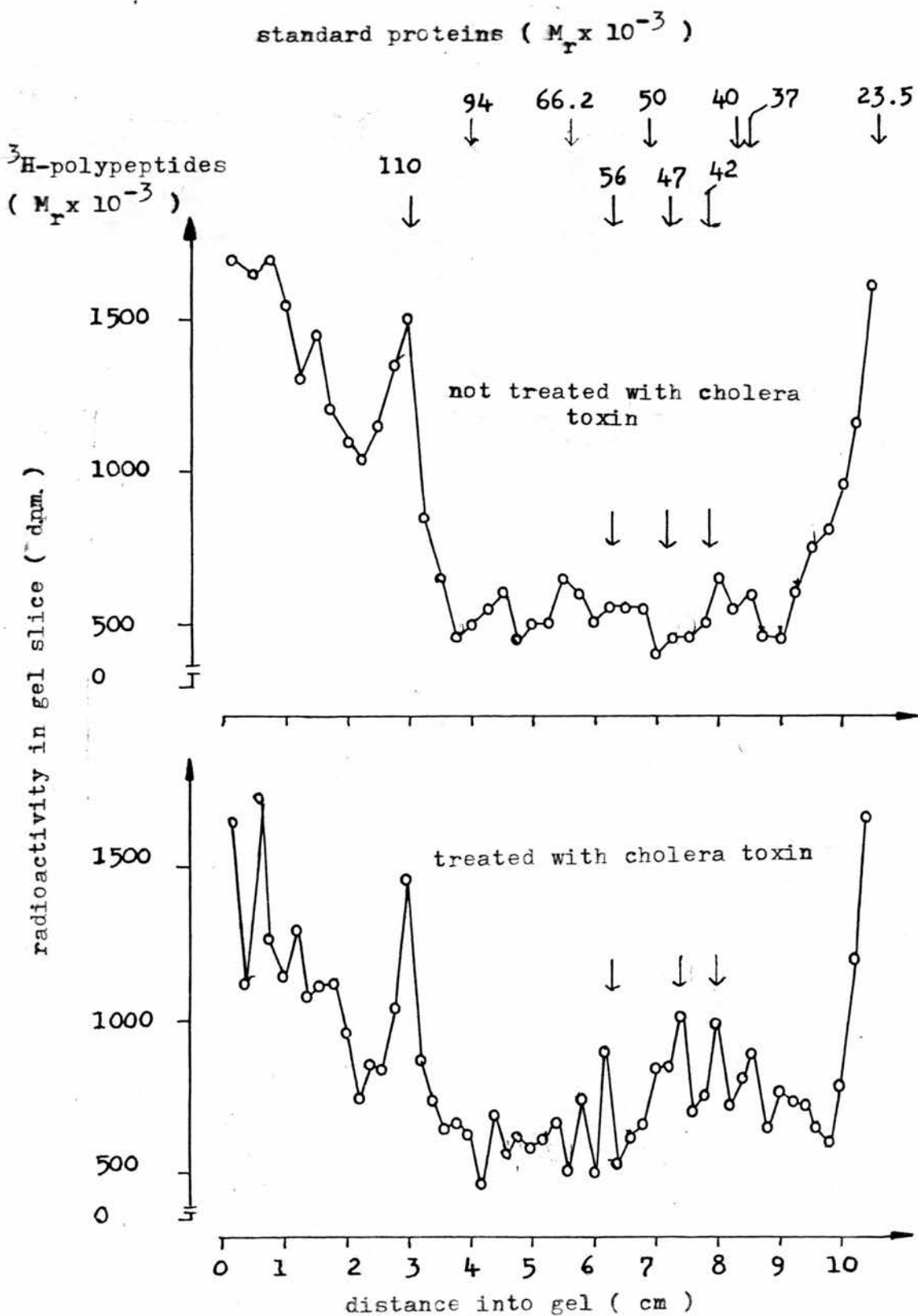
M_r values and
markers

110 000
← phosphorylase b
← BSA
← pyruvate kinase
← 56 000
← H-chain of immunoglobulin G
← 47 000
← 42 000 ← ovalbumin
← alcohol dehydrogenase

Fig. 4.2

Incorporation of radioactivity from $[^3\text{H}]\text{NAD}^+$ into membrane proteins

Rat liver membranes (about 0.5mg protein) were incubated with $[\text{adenosine-5',8-}^3\text{H}]\text{NAD}^+$ in the presence or absence of cholera toxin (section 2.6). Polypeptides were analysed by SDS-PAGE. The concentration of acrylamide in the separating gel was 8% (w/v). Gels were sliced and counted for radioactivity (section 2.8). The background number of d.p.m. have not been subtracted. Values of M_r were estimated as described in section 2.8. Similar results were obtained when the experiment was repeated a further twice.



for radioactivity (fig. 4.2). On some gels another substrate of cholera toxin of M_r about 39 000 could be detected. The infrequency of detection of this substrate suggested that it might have been produced by proteolysis of another radiolabelled molecule rather than being a genuine substrate of cholera toxin. The gel system that was used would only detect polypeptides of M_r 15 000 - 150 000. This system would not detect self-ADP-ribosylation by the A1 polypeptide of cholera toxin (as reported by Trepel et al., 1977), as the membranes were washed extensively before SDS-PAGE.

There was good agreement between the results of radiolabelling using $[^3H]NAD^+$ followed by gel slicing, with radiolabelling using $[^{32}P]NAD^+$ and then autoradiography. Polypeptides of M_r = 42 000 and 47 000 were shown to be substrates of cholera toxin, and were detected in similar amounts, using each technique (see figs 4.1 and 4.2, and table 4.17).

4.2.2 Requirements for the action of cholera toxin in rat liver membranes

4.2.2.1 The activation of adenylate cyclase

The effect of cholera toxin on the activity of adenylate cyclase required GTP and NAD^+ (fig. 4.3 and table 4.2). Using this preparation of membranes, cytosol was not required for the stimulation of adenylate cyclase by cholera toxin, but it did enhance both basal and toxin-stimulated activities to similar extents (table 4.3). The membrane preparation contained about 5% of the cytosolic material that was in the crude homogenate (fig. 2.1).

In these experiments, NAD^+ and GTP was added to the incubation with cholera toxin (see section 2.1) so that sufficient of these agents was present to allow maximal activation of adenylate cyclase by cholera toxin in the membrane preparation (figs. 4.3 and 4.9), and presumably also in the homogenate.

Fig. 4.3

The requirement for NAD^+ in the activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin in the presence of various concentrations of NAD^+ , washed, then assayed for adenylate cyclase activity (see sections 2.1 and 2.2). The values plotted below are means of duplicate determinations which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated.

adenylate cyclase
activity (pmol cAMP
produced / min / mg protein)

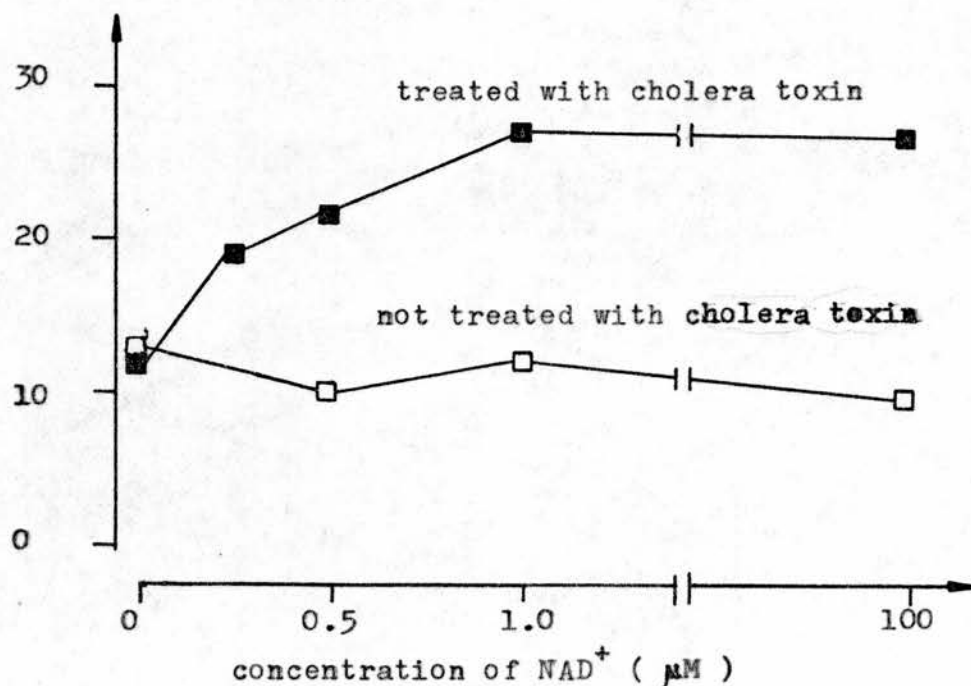


Table 4.2

Requirement for GTP in the activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin in the presence of various concentrations of ATP and GTP, washed and then assayed for activity of adenylate cyclase.

The adenylate cyclase activities given below are means of duplicate determinations which agreed to within $\pm 5\%$. Experimental details are given in sections 2.1 and 2.2. Similar results were obtained when the experiments were repeated.

<u>treatment with</u> <u>cholera toxin</u>	<u>concentration of:</u>		<u>adenylate cyclase</u> <u>activity (pmol cAMP</u> <u>produced/min/mg protein)</u>
	<u>GTP (mM)</u>	<u>ATP (mM)</u>	
-	0	0	26.1
+	0	0	26.6
-	0	1.5	28.5
+	0	1.5	31.4
-	0.3	0	25.2
+	0.3	0	43.5
-	2.0	0	26.2
+	2.0	0	49.4

Table 4.3

The effect of cytosol on the stimulation of adenylate cyclase by cholera toxin

The effect of cholera toxin was measured in a crude homogenate of rat liver and in a membrane preparation. The homogenate and the membranes were in the same buffer. The preparations were incubated with or without cholera toxin and then the membranes were washed before the activity of adenylate cyclase was assayed. The adenylate cyclase activities given are the means of duplicate determinations which agreed to within $\pm 5\%$. Experimental details are given in sections 2.1 and 2.2. Similar results were obtained when the experiments were repeated.

<u>preparation</u> <u>used</u>	<u>treatment with</u> <u>cholera toxin</u>	<u>cytosol</u>	<u>adenylate cyclase</u> <u>activity (pmol cAMP</u> <u>produced/min/mg</u> <u>protein)</u>	<u>activation</u> <u>of adenylate</u> <u>cyclase by</u> <u>cholera</u> <u>toxin</u>
membranes {	-	-	14.4	102%
	+	-	29.1	
homogenate {	-	+	18.6	88%
	+	+	35.0	

4.2.2.2 Requirements for radiolabelling of polypeptides by cholera toxin

Cholera toxin appeared to be transferring ADP-ribose from NAD^+ to polypeptides in rat liver membranes (see sections 4.2.3 and 4.3.3). The effect of adding cytosol on radiolabelling by cholera toxin could not be tested as the presence of NAD^+ in the cytosol would decrease the specific activity of radioactive NAD^+ . Cytosol which had been pretreated with an insoluble NADase could have been tested. The labelling of membrane proteins by cholera toxin and $[\text{adenylate-}^{32}\text{P}]\text{NAD}^+$ was not dependant upon cytosol as the preparation of membranes did not contain cytosol (see fig. 2.1). The requirement for NAD^+ in the activation of adenylate cyclase by cholera toxin correlated with the transfer of radiolabel from NAD^+ to membrane proteins by cholera toxin. The amount of radiolabel incorporated into each of the substrates of cholera toxin increased with increasing concentration of NAD^+ up to about 10 μM (fig. 4.4). GTP was required for ADP-ribosylation by cholera toxin (table 4.13).

4.2.3 Evidence for the action of cholera toxin involving ADP-ribosylation

4.2.3.1 The activation of adenylate cyclase

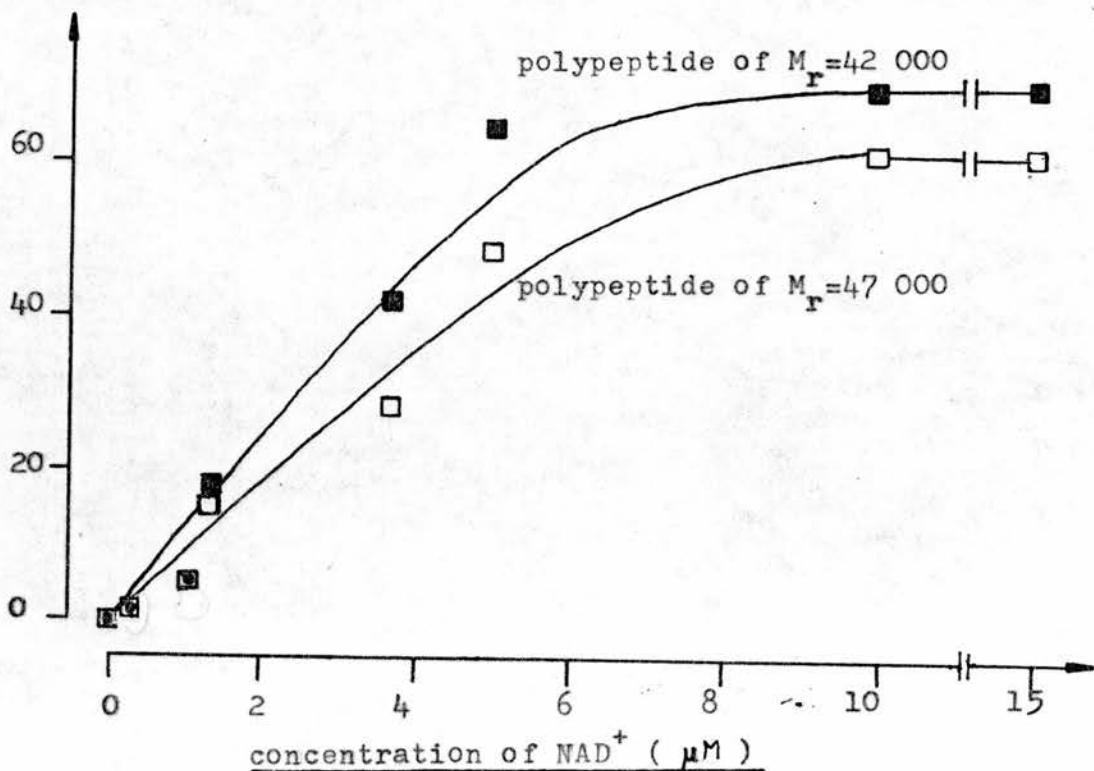
Work in other systems suggested that the action of cholera toxin involved ADP-ribosylation (see section 1.3.3). The requirement for NAD^+ which was found in the current study (fig. 4.3) implied that ADP-ribosylation could also be involved here. ADP-ribosylation using NAD^+ as the donor would produce nicotinamide (fig. 1.7). Therefore the inclusion of nicotinamide in the incubation with cholera toxin could change the position of the equilibrium to inhibit ADP-ribosylation.

Fig. 4.4

Labelling of membrane polypeptides by cholera toxin at various concentrations of [adenylate- ^{32}P] NAD^+

Rat liver membranes were incubated with cholera toxin in the presence of various concentrations of [^{32}P] NAD^+ (see section 2.6). The extent of radiolabelling of polypeptides was determined as described in section 2.9.3 . The values are single determinations only ,but similar results were obtained when the experiment was repeated . In the absence of cholera toxin less than 2fmol ADP-ribose per mg membrane protein were incorporated into each polypeptide . 0.5mg membrane protein was used in each assay of ADP-ribosylation.

incorporation of ADP-ribose
(fmol / mg membrane protein)



Low concentrations of nicotinamide enhanced the activation of adenylate cyclase by cholera toxin. This was probably due to inhibition of other enzymes which degrade NAD^+ . Increasing the concentration of nicotinamide progressively inhibited the action of cholera toxin (fig. 4.5). This effect was specific for the activation of adenylate cyclase by cholera toxin. Stimulation of the enzyme by fluoride ions was not affected (see section 2.2 and fig. 2.7). The influence of nicotinamide was not due to ionic strength (see section 2.2.3). These results confirmed those of Tait (1980).

NAD^+ -dependant ADP-ribosylation by cholera toxin would produce protons and nicotinamide (fig. 1.7). The reaction has been reversed in avian erythrocytes by incubation at low pH, high nicotinamide concentration and low NAD^+ concentration. Cholera toxin catalysed this reversal (Gill and Meren, 1978). In the current work stimulation by fluoride ions was used to test the stability of adenylate cyclase activity (see section 2.2). Rat liver membranes were incubated in the presence or absence of cholera toxin and 20mM NaF , washed and then treated as described in section 4.1.2. The aim of this second incubation was to reverse the activation of adenylate cyclase by changing the position of the equilibrium for ADP-ribosylation as described above. The membranes were washed and then assayed for the activity of adenylate cyclase. However, the enzyme was labile to the conditions of the reversal incubation (table 4.4). All of the decreases in cholera toxin-stimulated adenylate cyclase activity were smaller than the corresponding decreases in fluoride-stimulated activity and therefore could be accounted for as non-specific inactivation. This result for the rat liver system was confirmed by Doberska et al. (1980).

Fig. 4.5

The effect of nicotinamide on the activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin in the presence of various concentrations of nicotinamide, washed and then assayed for activity of adenylate cyclase. The values given for adenylate cyclase activities are means of duplicate determinations which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated. Experimental details are given in sections 2.1 and 2.2.

adenylate cyclase
activity (pmol cAMP produced/
min/mg protein)

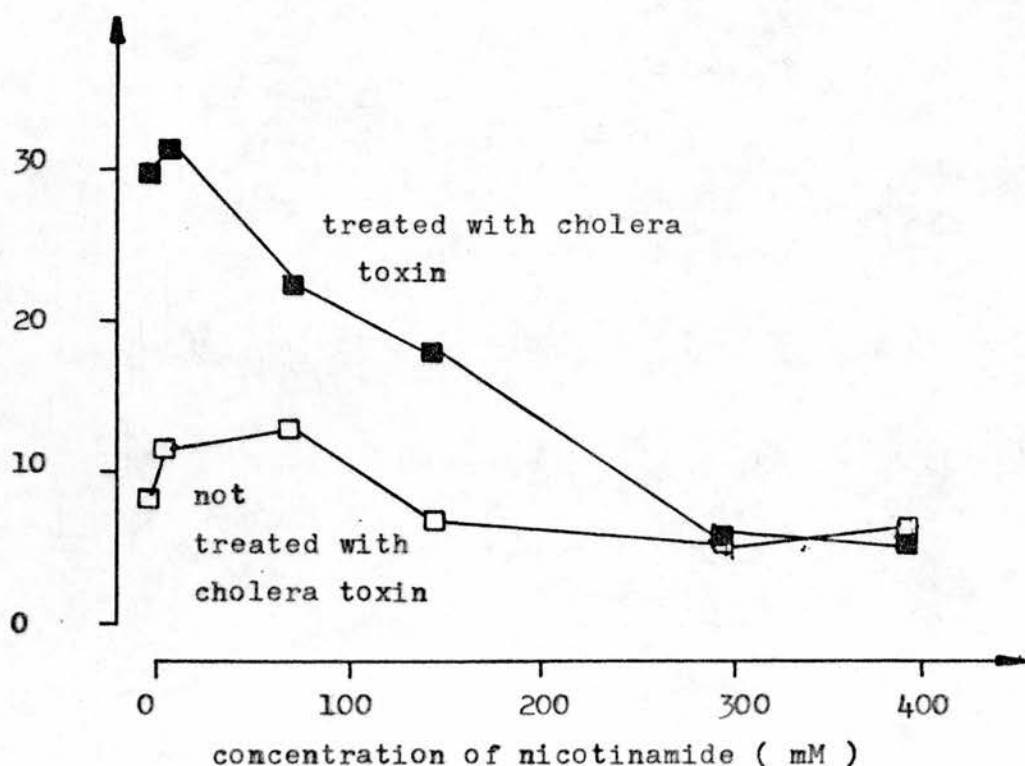


Table 4.4

Attempted reversal of the activation of adenylate cyclase by cholera toxin

Rat liver membranes were treated as described in the text (section 4.2.3.1). The adenylate cyclase activities are means of duplicate determinations which agreed to within $\pm 5\%$. Experimental details are given in sections 2.1, 2.2 and 4.1.2. Membranes were treated with reversal medium of each pH in two different experiments. All of the pH's were not tested each time. Similar results were obtained when the experiments were repeated.

<u>type of adenylate cyclase activity</u>	<u>adenylate cyclase activity (pmol cAMP produced/min/mg/protein)</u>			
	<u>pH of reversal medium</u>			
	<u>6.4</u>	<u>6.6</u>	<u>6.9</u>	<u>7.2</u>
experiment	i	ii	iii	iii
basal	7.9	14.4	6.8	6.8
stimulated by cholera toxin	14.2	29.1	22.7	22.7
stimulated by toxin and fluoride*	140.2	126.2	168.8	168.8
stimulated by toxin then incubated with reversal medium	14.1	14.8	20.3	19.0
% decrease in toxin-stimulated adenylate cyclase activity	0.7	49.1	10.6	16.3
stimulated by toxin then incubated with reversal medium containing fluoride to 20mM	17.8	33.4	76.4	62.6
% decrease in fluoride-stimulated adenylate cyclase activity	87.3	73.5	54.7	62.9

*Pretreatment with cholera toxin decreases the sensitivity of adenylate cyclase to fluoride, therefore control membranes were treated with both activators.

4.2.3.2 Evidence for ADP-ribosylation of polypeptides by cholera toxin

Radioactivity was transferred from [γ -adenylate- ^{32}P]/ NAD^+ or [γ -adenosine-5', 8- ^3H]/ NAD^+ to membrane proteins by cholera toxin (figs 4.1 and 4.2). The positions of the labelling groups from the NAD^+ molecule were consistent with the occurrence of ADP-ribosylation. NAD^+ which was radioactive in its nicotinamide moiety was not tested for the ability to support labelling of polypeptides by cholera toxin, but the inhibition of cholera toxin-specific labelling by nicotinamide (table 4.5) suggested that cholera toxin was not attaching the whole of the NAD^+ molecule to proteins. Nicotinamide blocked the cholera toxin-specific radiolabelling of polypeptides using [γ -adenylate- ^{32}P]/ NAD^+ . The endogenous labelling of only some of the polypeptides was inhibited (table 4.5). This was not an effect of ionic strength as NaCl failed to block the action of cholera toxin (table 4.5 and section 2.2.3). These results were confirmed by Doberska *et al.* (1980).

The labelling of the substrates of cholera toxin was not significantly reversed by incubation at low pH and high concentrations of nicotinamide in the presence of toxin (table 4.5). Similar observations were made using plasma membranes from rat liver (Doberska *et al.*, 1980). These data were in agreement with the results of attempts to reverse the activation of adenylyl cyclase by cholera toxin (section 4.2.3.1).

The results of the current work were consistent with cholera toxin transferring ADP-ribose groups from NAD^+ to membrane proteins, but they failed to prove that this was the case. When these data are taken together with the results of work done in other systems (see section 1.3.3), it seems almost certain that cholera toxin was acting as an ADP-ribosyl-transferase in the current work.

Table 4.5

Attempted demonstration of the transfer of ADP-ribose from
 γ -adenylate- ^{32}P /NAD $^{+}$ to membrane proteins

Rat liver membranes were incubated with cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$ as described in section 2.6. The extent of radiolabelling of polypeptides was measured by autoradiography (see section 2.9.3). Reversal incubations were carried out as described in section 4.1.2. The values are single determinations only, but similar results were obtained when the experiments were repeated. 0.5mg membrane protein was used in each ADP-ribosylation assay.

<u>treatment of membranes</u>	<u>incorporation of radiolabel</u> <u>(fmol/mg membrane protein)</u> <u>into polypeptides of M_r</u>		
<u>experiment (i)</u>	<u>110 000</u>	<u>47 000</u>	<u>42 000</u>
control (no toxin added)	36	<2	<2
with cholera toxin	36	19.5	33
incubation with 300mM nicotinamide but in the absence of toxin	66	<2	<2
incubation with toxin and 300mM nicotinamide	66	<2	<2
<u>experiment (ii)</u>			
control (no toxin added)	35	<2	<2
with cholera toxin	27	60	71
with cholera toxin, then reversal incubation at:			
pH 6.4	27	57	74
pH 6.6	27	60	63
pH 6.9	30	54	68
pH 7.2	24	59	71
pH 6.9 but with no toxin in the reversal medium	27	49	60

4.2.4 Evidence for the modification of arginine or arginine-like residues by cholera toxin

Arginine or other compounds containing guanidinium groups stimulate the ability of preparations of cholera toxin to catalyse the release of nicotinamide from NAD^+ (Moss & Vaughan, 1977). N.m.r. studies showed that cholera toxin catalysed the ADP-ribosylation of arginine (Oppenheimer, 1978). However, there is little evidence to suggest that cholera toxin interacts with arginine residues in proteins (see section 4.3.4). The abilities of compounds that are related to arginine to inhibit the action of cholera toxin was therefore investigated.

4.2.4.1 Modification of arginine or arginine-like residues in the activation of adenylate cyclase

The pK of the guanidino-group in arginine is higher than the pK of the guanidino-group of arginine residues in polypeptides. Esterification of arginine causes the pK of the guanidino-group to become similar to that when the group occurs on a side-chain in polypeptides. Therefore arginine methyl ester was used as an analogue of arginine residues in proteins. Low concentrations of arginine methyl ester increased basal and cholera toxin-stimulated adenylate cyclase activities (fig. 4.6) (this was an effect of ionic strength, see section 2.2.3). High concentrations of the compound blocked the activation of adenylate cyclase by cholera toxin (fig. 4.6). This inhibition was specific as the stimulation of the enzyme by fluoride ions was not affected (fig. 2.7).

Compounds which are structurally related to arginine methyl ester were tested for the ability to block the action of cholera toxin (table 4.6). Such compounds could have affected the stability of adenylate cyclase. The ratio of stimulation of adenylate cyclase by cholera toxin to stimulation by fluoride ions was therefore used as a measure of the ability

Fig. 4.6

The effect of arginine methyl ester on the activation of
adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin in the presence of various concentrations of arginine methyl ester, washed and then assayed for activity of adenylate cyclase. The values which are shown are the means of duplicate determinations which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated. Experimental details are given in sections 2.1 and 2.2.

adenylate cyclase
activity (pmol cAMP produced/
min/mg protein)

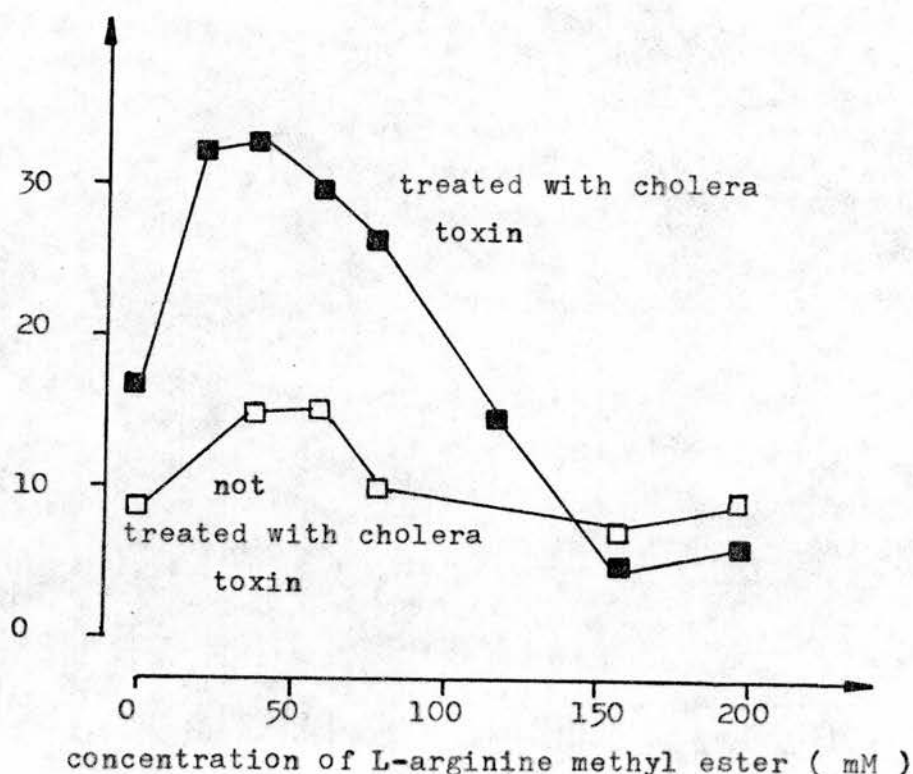


Table 4.6

The effects of arginine methyl ester and related compounds on the activation of adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin in the presence of various compounds, washed and then assayed for activity of adenylate cyclase. The reasoning behind the use of NaF to measure the stability of adenylate cyclase is given in section 4.2.4.1. The values given for the activities of adenylate cyclase are means of duplicates which agreed to within $\pm 5\%$. Similar results were obtained when the experiments were repeated. Experimental details are given in sections 2.1 and 2.2.

<u>addition</u>	<u>adenylate cyclase activity (pmol cAMP produced/min/mg protein)</u>			
	<u>basal</u>	<u>stimulated</u>	<u>stimulated</u>	<u>(b)-(a)</u>
		<u>by toxin</u>	<u>by fluoride</u>	
	(a)	(b)	(c)	(c)-(a)
experiment (i)				
none	11.4	22.4	149.2	0.08
250mM NaCl	10.0	19.0	139.0	0.07
150mM L-arginine methyl ester	14.9	14.4	149.1	≤ 0
150mM guanidine	6.0	5.1	42.7	≤ 0
150mM L-lysine methyl ester	9.0	13.8	127.8	0.04
150mM L-citrulline	11.4	20.3	135.0	0.07
experiment (ii)				
none	15.8	39.4	134.0	0.20
150mM L-histidine	15.6	37.0	149.6	0.16
150mM L-arginine methyl ester	15.8	15.6	131.0	≤ 0

to specifically inhibit the action of cholera toxin. Incubation with 20mM NaF followed by washing and then assay of adenylate cyclase activity was used to measure the activity of the regulatory and catalytic components of the enzyme (see section 1.3.2). Guanidine blocked the effect of cholera toxin, but it caused a 71% loss in fluoride ion-stimulated adenylate cyclase activity. This was due to an inactivating effect on adenylate cyclase and possibly also on cholera toxin. At concentrations where arginine methyl ester completely blocked the action of cholera toxin, lysine methyl ester only partially inhibited the effect of the toxin. Citrulline differs from arginine only in having a ureido group rather than a guanidino-group. But, at concentrations where arginine methyl ester abolished stimulation by cholera toxin, citrulline had no significant effect on the activation of adenylate cyclase by the toxin.

Phenylglyoxal reacts highly specifically with arginine residues in proteins (Takahashi, 1968). In the current work it was attempted to block the effect of cholera toxin on adenylate cyclase by preincubating the membranes with phenylglyoxal. These experiments were not successful as the regulatory component of adenylate cyclase was more sensitive than the catalytic protein to inactivation by phenylglyoxal (see section 4.2.5.1).

4.2.4.2 ADP-ribosylation of arginine, or arginine-like, residues by cholera toxin

High concentrations of arginine methyl ester blocked cholera toxin-specific, but not endogenous, labelling of rat liver membrane proteins using γ -adenylate- ^{32}P /NAD $^{+}$ (table 4.7). This was not an effect of ionic strength as it could not be mimicked by NaCl (table 4.7). Citrulline (which is closely related to arginine in structure) also failed to block the action of cholera toxin (table 4.7). Lysine methyl ester, like arginine methyl ester, possesses a basic nitrogen atom

Table 4.7

The effects of arginine methyl ester and related compounds on ADP-ribosylation by cholera toxin

Rat liver membranes were incubated with or without cholera toxin in the presence of γ -adenylate- ^{32}P /NAD $^{+}$ and various other compounds, washed and then assayed for the amount of ADP-ribosylation which had occurred. The values are single determinations only, but similar results were obtained when the experiments were repeated. Experimental details are given in sections 2.6 and 2.9.3. 0.5mg protein was used in each ADP-ribosylation assay.

<u>treatment of membranes</u>		<u>incorporation of ADP-ribose</u> <u>(fmol/mg membrane protein)</u>		
<u>additions to</u> <u>incubation</u>	<u>treatment with</u> <u>cholera toxin</u>	<u>into polypeptides of M_r</u>		
		<u>110 000</u>	<u>47 000</u>	<u>42 000</u>
none	-	36	<2	<2
none	+	36	20	33
250mM NaCl	-	11	<2	<2
250mM NaCl	+	11	25	36
150mM L-arginine methyl ester	-	9	<2	<2
150mM L-arginine methyl ester	+	8	<2	<2
150mM L-lysine methyl ester	-	<2	<2	<2
150mM L-lysine methyl ester	+	<2	<2	<2
150mM L-citrulline	-	11	<2	<2
150mM L-citrulline	+	13	26	40

at the end of a hydrocarbon chain. Lysine methyl ester was the only other amino-acid or related compound which blocked ADP-ribosylation by cholera toxin (table 4.7). This suggested that a basic nitrogen atom is the acceptor of the labelling group. High ionic strength partially inhibited the endogenous labelling of a polypeptide of $M_r \approx 110\ 000$. Incorporation of radioactivity into this molecule was completely blocked by lysine methyl ester (table 4.7). The effects of different compounds on the activation of adenylate cyclase by cholera toxin paralleled the effects on ADP-ribosylation (see tables 4.6 and 4.7). Slight effects of cholera toxin were more easily detected in the adenylate cyclase assay than in the ADP-ribosylation assay as the former system produces a catalytically active species (see section 4.3.4).

4.2.5 Evidence for the functional modification of the regulatory component of adenylate cyclase by cholera toxin

4.2.5.1 The activation of adenylate cyclase by cholera toxin

Functional modification of a factor which regulates adenylate cyclase by cholera toxin

Rat liver membranes which had been freshly prepared were much more sensitive than stored membranes to the action of cholera toxin, but stored membranes retained much of their fluoride-stimulated adenylate cyclase activity (see table 4.8. Membranes were stored at -70°C). Stored membranes probably had low adenylate cyclase activity because freezing and thawing caused rupture of lysosomal membranes and release of hydrolytic enzymes. (Lysosomes were present in the preparation, see fig. 2.1.) Adenylate cyclase could also be labile to the physical effects of freezing and thawing, especially with respect to susceptibility to cholera toxin. It is surprising that freezing and thawing had a differential effect on the sensitivity of adenylate cyclase to fluoride

Table 4.8

Properties of adenylate cyclase in freshly prepared, and stored, rat liver membranes

Rat liver membranes were incubated with or without cholera toxin or 20mM NaF, washed and then assayed for activity of adenylate cyclase. The values for activities of the enzyme are means of duplicates which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated. Experimental details are given in sections 2.1 and 2.2.

	<u>adenylate cyclase activity</u> (pmol cAMP produced/min/mg protein)			
	<u>basal</u>	<u>stimulated</u> <u>by toxin</u>	<u>stimulated</u> <u>by fluoride</u>	<u>relative</u> <u>sensitivity</u> <u>to toxin*</u>
freshly prepared membranes	8.3	18.3	175.0	0.060
stored membranes	4.8	6.5	82.9	0.022

*Ratio of stimulation of adenylate cyclase by cholera toxin to stimulation by fluoride.

ions and sensitivity to cholera toxin, as both agents act through the same regulatory protein (see section 1.3.3).

To test if cholera toxin modified a regulatory factor in the adenylate cyclase complex, sensitive (fresh) membranes were treated with toxin. The membranes were washed then solubilized. The extract was added to relatively insensitive (stored) membranes and then the adenylate cyclase activity of the mixture was measured. Recipient membranes which had been complemented with extract from cholera toxin-treated membranes had higher adenylate cyclase activity than membranes which had been treated with control extracts (tables 4.9 and 4.10). Carry-over of cholera toxin could not account for this difference (tables 4.9 and 4.10). The adenylate cyclase activity of the complemented mixture was higher than the sum of that of its constituent parts (table 4.10). This showed that cholera toxin was modifying a regulatory factor, rather than a catalytic protein in the adenylate cyclase complex.

The complementation experiments were performed using small amounts of protein in the adenylate cyclase assay (because detergent had to be diluted in the complementation mixture for the stability of catalytic activity). In these experiments adenylate cyclase was assayed at 15°C because the enzyme is very sensitive to temperature after solubilization from rat liver (van Heyningen, 1976b). Therefore only small amounts of cAMP were produced in the assays of adenylate cyclase. Differences were amplified by expressing the activity per mg protein. The measured differences, although small, were consistent. The inhibitory effect of high concentrations of detergent and the low adenylate cyclase activities in the assays of the complemented mixtures limited the number of data points which could be used in these experiments.

Table 4.9

Complementation studies of adenylate cyclase activity

Freshly prepared rat liver membranes were incubated with, or without, cholera toxin, washed and then solubilized by method (a) in section 4.1.4. The detergent extracts were complemented with stored membranes in a final volume of 100 μ l (see sections 4.1.5 and 4.2.5.1). Then the adenylate cyclase activities in duplicate 25 μ l samples were determined. The mean values of the duplicates (which agreed to within $\pm 10\%$) are given here. Similar results were obtained when the experiment was repeated. Experimental details are given in sections 2.1 and 2.2.

<u>donor (fresh) membrane</u> <u>extract</u>		<u>recipient (stored)</u> <u>membranes</u>	<u>adenylate cyclase</u> <u>activity</u>
<u>treatment with</u> <u>toxin</u>	<u>protein</u> <u>(mg)</u>	<u>protein</u> <u>(mg)</u>	<u>(pmol cAMP produced/</u> <u>min/mg donor protein)</u>
-	0.09	0	6.4
-	0.09	0.18	27.2
+	0.09	0	17.6
+	0.09	0.18	64.0
+	0*	0.38	1.6

*No extract from fresh membranes was added to the complementation mixture. Buffer was treated with cholera toxin and detergent to test for carry-over of toxin in solution. For these samples the adenylate cyclase activity is quoted per mg recipient protein.

Table 4.10

Complementation studies of adenylate cyclase activity

Freshly prepared rat liver membranes were incubated with cholera toxin, washed and then solubilized by method (b) in section 4.1.4. The detergent extracts were complemented with stored membranes in a final volume of 100 μ l (see sections 4.1.5 and 4.2.5.1). Then the adenylate cyclase activities in duplicate 25 μ l samples were determined. The mean values of the duplicates (which agreed to within $\pm 10\%$) are given here. Similar results were obtained when the experiment was repeated. Experimental details are given in sections 2.1 and 2.2.

<u>recipient (stored)</u> <u>membranes</u>	<u>protein (mg)</u>	<u>adenylate cyclase activity (pmol</u>			
	<u>donor (fresh)</u>	<u>cAMP produced/min/mg donor protein)</u>			
	<u>membrane</u> <u>extract</u>	<u>observed</u>	<u>expected</u>		
			<u>recipient</u>	<u>donor</u>	<u>sum</u>
0	0.038	1.6	0	1.6	1.6
0.0035	0.038	4.8	0.6	1.6	2.2
0.0070	0.038	11.2	1.2	1.6	2.8
0.19	0 ¹	4.0			
0.19	0 ²	6.4			

1 & 2 No fresh membrane extract was added to the complementation mixture. Buffer was treated with cholera toxin and detergent to test for carry-over of toxin in solution. No cholera toxin was added to ¹. For these samples the adenylate cyclase activities are quoted per mg recipient protein.

Functional modification of a protein which binds guanyl nucleotides by cholera toxin

Membrane preparations which had been stored were less sensitive than freshly prepared membranes to activation of adenylate cyclase by p(NH)ppG (fig. 4.7). There was also variation between rats in sensitivity to cholera toxin or p(NH)ppG. The correlation between the susceptibility of different preparations to the two stimulators was statistically significant (i.e. had a probability of 1-5% of arising by chance alone (see appendix II); correlation coefficient = 0.98, four pairs of observations) (fig. 4.7). This suggested that p(NH)ppG and cholera toxin activated the enzyme via the same regulatory mechanism. The percent increase in adenylate cyclase activity above basal levels was used for these comparisons (see fig. 4.7), to allow for differences in catalytic activities between the preparations. Manganese ion-stimulated activity of adenylate cyclase would have been a better measure than basal activity of the catalytic ability of the membranes, as manganese ions act directly on the catalytic component of the enzyme (Ross & Gilman, 1980). But this was not known when the experiments were done in the current work.

Adenylate cyclase which had been activated by p(NH)ppG could not be further stimulated by cholera toxin (table 4.11). This suggested that the two agents acted on the same regulators of adenylate cyclase activity. Treatment with the stimulators individually showed that cholera toxin was less potent than p(NH)ppG (table 4.11).

Phenylglyoxal inactivates proteins by reacting with arginine residues (Takahashi, 1968; Franks et al., 1980). Pretreatment of membranes from rat liver with phenylglyoxal decreased the activity of adenylate cyclase. If membranes were subsequently treated with cholera toxin, the adenylate cyclase activity was reduced to a relatively greater extent than basal activity

Fig. 4.7

Correlation between the sensitivities of adenylate cyclase to stimulation by cholera toxin or p(NH)ppG

Rat liver membranes were treated with p(NH)ppG to 1mM or with cholera toxin, washed and then the increase above basal adenylate cyclase activity was determined. Each point represents a result for a different preparation of membranes. The values quoted are the means of duplicates. Determinations of adenylate cyclase activities agreed to within $\pm 5\%$. The estimates of the errors in the individual data points are shown in the figure. Experimental details are given in sections 2.1 and 2.2.

■, freshly prepared membranes. □, stored membranes.

percent stimulation above
basal adenylate cyclase
activity by p(NH)ppG

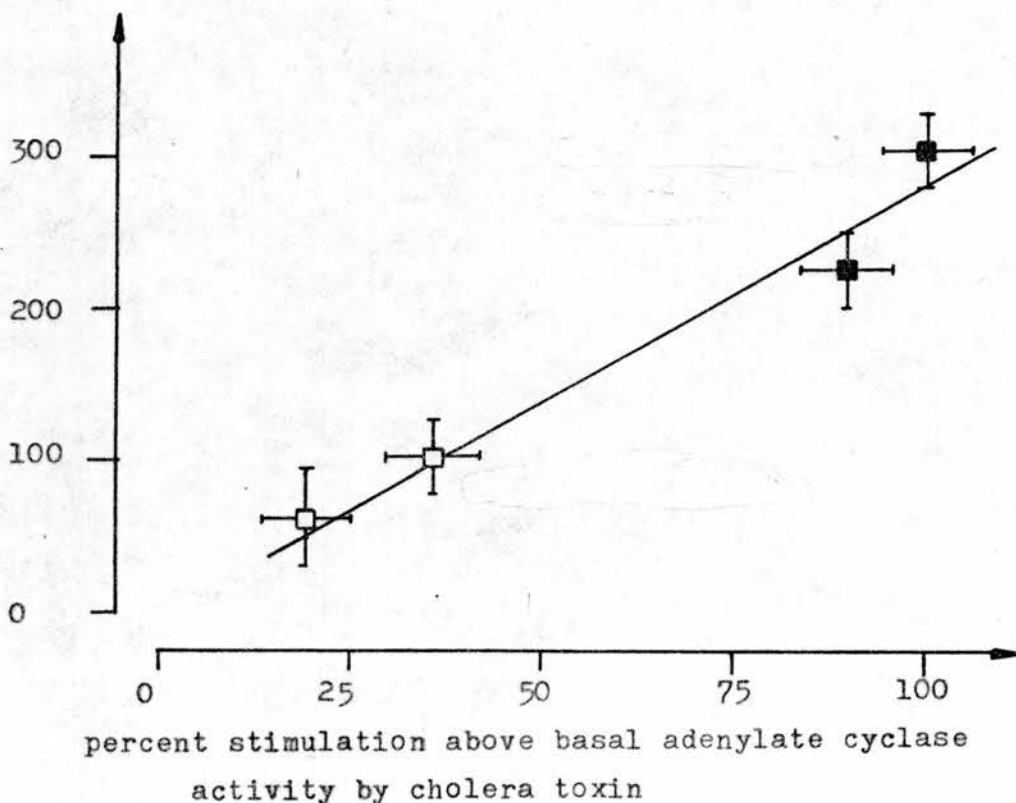


Table 4.11

The stimulation of adenylate cyclase by cholera toxin and p(NH)ppG

Rat liver membranes were incubated with, or without, cholera toxin and 1mM p(NH)ppG, washed and then assayed for activity of adenylate cyclase. The values given below are means of duplicates which agreed to within $\pm 5\%$. Similar results were obtained when the experiment was repeated.

<u>stimulators of</u> <u>adenylate cyclase</u>	<u>adenylate cyclase activity</u> <u>(pmol cAMP produced/min/mg protein)</u>
none	9.0
1mM p(NH)ppG	33.6
cholera toxin	19.0
1mM p(NH)ppG and cholera toxin	36.5

was reduced (fig. 4.8). Stimulation by p(NH)ppG was also inhibited by pretreatment with phenylglyoxal, suggesting that cholera toxin and p(NH)ppG affect the same regulatory factor (fig. 4.8). Phenylglyoxal could be useful in preparing adenylate cyclase which is deficient in its regulatory function like that of the mouse S49 cyc⁻ lymphoma (Johnson *et al.*, 1978). Phenylglyoxal may cause inactivation by reacting with essential arginine residues, or because of producing steric hindrance or conformation changes. The stability of basal adenylate cyclase activity to phenylglyoxal suggested that the reagent did not react readily with the catalytic moiety of the enzyme. This hypothesis could be tested by measuring the manganese ion-stimulated activity of adenylate cyclase (see above). The non-linear nature of the profile for inactivation by phenylglyoxal (fig. 4.8) suggested that more than one (probably two) arginine residues were being modified. These results are in close agreement with those subsequently published by Tait (1981).

A substrate analogue (p(NH)ppG) failed to protect the GTPase which regulates adenylate cyclase from inactivation by phenylglyoxal.

Pretreatment of the membranes with cholera toxin or p(NH)ppG increased the susceptibility of adenylate cyclase to inactivation by phenylglyoxal (table 4.12). Activators of the enzyme apparently induced increased exposure of target residues. This may be due to changes in conformation or disaggregation of the adenylate cyclase complex. Cholera toxin and p(NH)ppG again had similar effects.

Attempted demonstration of inhibition of a regulatory GTPase by cholera toxin

Cholera toxin inhibited a regulatory GTPase in avian erythrocyte membranes. This induced an increase in the activity of

Fig. 4.8

The effect of pretreatment with phenylglyoxal on the activity of adenylate cyclase

Rat liver membranes were incubated with phenylglyoxal (see section 4.1.3) and then re-equilibrated in perfusion buffer by washing in it four times. After a second incubation with or without cholera toxin or 1mM p(NH)ppG the membranes were washed and then the activities of adenylate cyclase were measured. Duplicate determinations agreed to within $\pm 5\%$. Adenylate cyclase activities after pretreatment at zero phenylglyoxal concentration were taken as 100. The means of duplicate values are given below. Duplicate data points agreed to within $\pm 10\%$ except for the highest concentration of phenylglyoxal where agreement was within $\pm 20\%$. Experimental details are given in sections 2.1 and 2.2. Similar results were obtained when the experiment was repeated.

relative adenylate cyclase activity

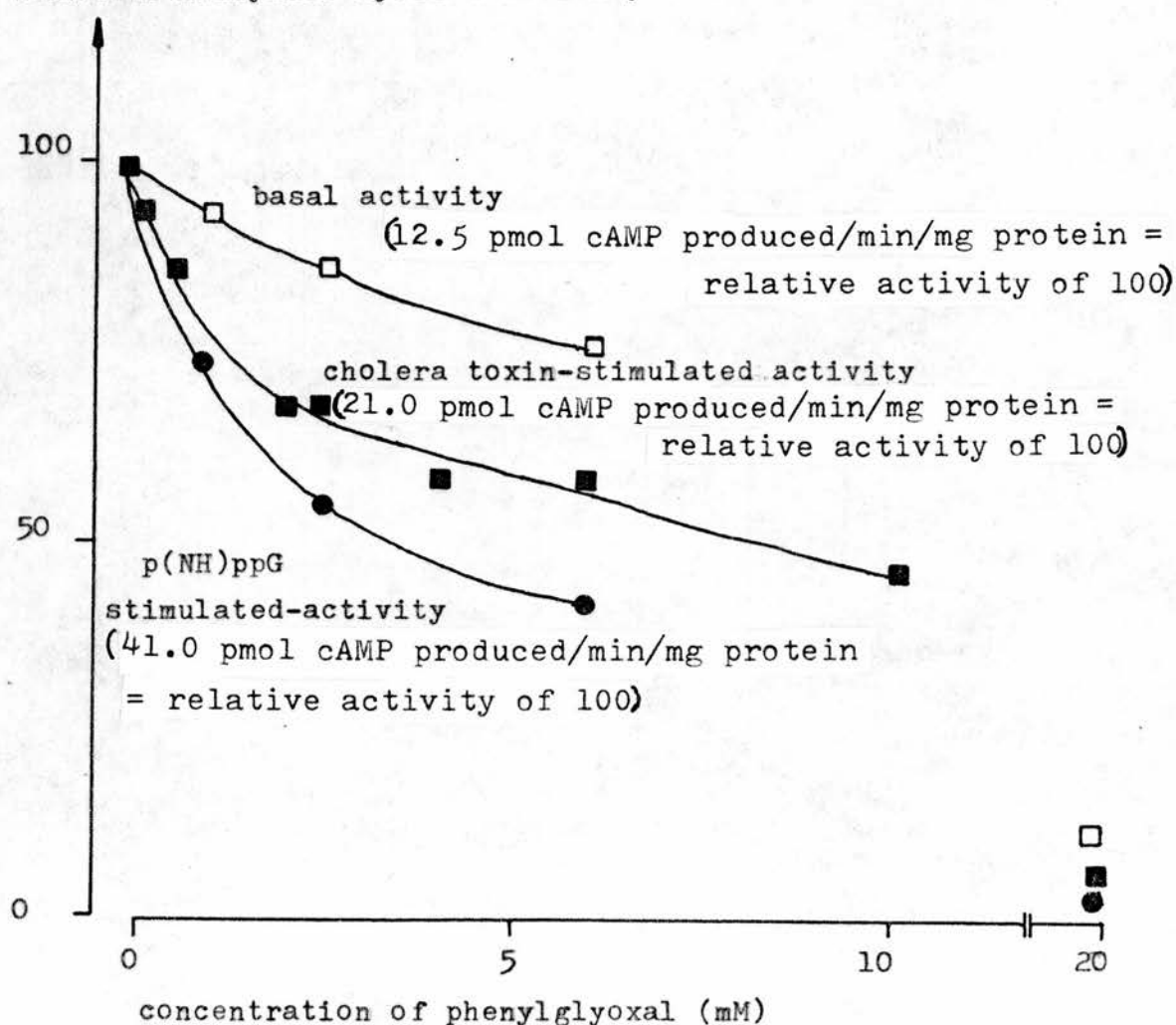


Table 4.12

The effects of pretreatment with cholera toxin or p(NH)ppG on the inactivation of adenylate cyclase by phenylglyoxal

Rat liver membranes were incubated with cholera toxin or 1mM p(NH)ppG, or alternatively treated with phenylglyoxal. Membranes were then washed three times and then subjected to the incubation which they had not yet received. Membranes were washed a further three times then adenylate cyclase activities were assayed. The mean values of duplicates which agreed within $\pm 5\%$ are quoted. Similar results were obtained when the experiment was repeated. Experimental details are given in sections 4.1.3, 2.1 and 2.2.

<u>treatment</u>	<u>adenylate cyclase activity (pmol cAMP produced/min/mg protein)</u>	
	<u>0.5mM phenylglyoxal</u>	<u>2.0mM phenylglyoxal</u>
p(NH)ppG then phenylglyoxal	8.0	5.6
phenylglyoxal then p(NH)ppG	19.8	10.0
toxin then phenylglyoxal	7.6	1.4
phenylglyoxal then toxin	18.2	4.5

adenylate cyclase (Cassel & Selinger, 1977, see section 1.3.3). Workers using plasma membranes purified from rat liver failed to demonstrate a regulatory GTPase activity as the preparation was contaminated with other GTPases (Doberska et al., 1980). Therefore it would be unlikely that a regulatory GTPase could be detected in the crude membrane system which was used in the current work. In an attempt to detect inhibition of a regulatory GTPase by cholera toxin, the concentration of GTP in the adenylylase assays was varied from 10 μ M to 0.41mM. But there was no significant change in basal or cholera toxin-stimulated activity of the enzyme (fig. 4.9) showing that, in this range, the concentration of GTP was not limiting adenylylase activity. Even at very high concentrations of GTP (0.41mM) the effect of pretreatment with cholera toxin could be seen (fig. 4.9), showing that its effect was not due to GTP limiting adenylylase activity in control membranes but not in cholera toxin-treated membranes.

4.2.5.2 ADP-ribosylation of polypeptides in the regulatory component of adenylylase

The observed requirement for GTP (table 4.13) is consistent with the suggestion that cholera toxin ADP-ribosylates the regulatory component of adenylylase. Fresh and stored membranes differed in susceptibility to stimulation of adenylylase by cholera toxin or p(NH)ppG (fig. 4.7). Membrane preparations varied in parallel in the amounts they were ADP-ribosylated by cholera toxin (table 4.13). Treatment with detergent solubilized factors with the ability to regulate adenylylase activity from membranes which had been preincubated with cholera toxin (section 4.2.5.1). The same treatment solubilized much of the protein which was ADP-ribosylated by cholera toxin (table 4.13). In rat liver, adenylylase occurs only in the plasma membranes (Wattiaux-De Coninck et al., 1981). In the current work it

Fig 4.9

The effect of varying the concentration of GTP on the activity of adenylate cyclase

Rat liver membranes were incubated with or without cholera toxin, washed and then the activities of adenylate cyclase were measured. The values given are the means of duplicate determinations which agreed to within $\pm 5\%$. The concentration of GTP in the assays of adenylate cyclase was varied. Similar results were obtained when the experiment was repeated. Experimental details are given in sections 2.1 and 2.2.

adenylate cyclase
activity (pmol cAMP
produced/min/mg protein)

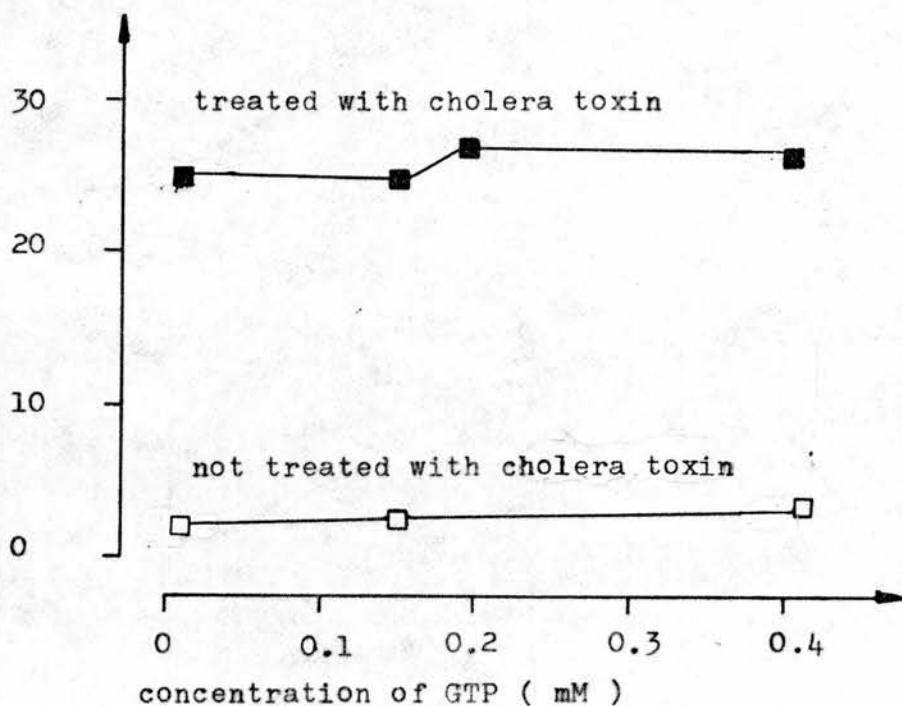


Table 4.13

Evidence for the ADP-ribosylation of the regulatory component of
adenylate cyclase by cholera toxin

Rat liver membranes were incubated with or without cholera toxin in the presence of γ -adenylate- ^{32}P /NAD $^{+}$ washed and then assayed for the amount of ADP-ribosylation which had occurred (see sections 2.6 and 2.9.3). The values are single determinations only, but similar results were obtained when the experiments were repeated. 0.5mg membrane protein was used in each ADP-ribosylation assay.

s indicates that membranes were stored at -70°C before use. f shows that freshly prepared membranes were used. * after incubation with cholera toxin and washing, membranes were solubilized by method (b) in section 4.1.4. In experiment *, 1 the insoluble pellet was analysed and in experiment *, 2 the supernatant was used.

experiment	type of membranes	treatment with toxin	concentration of:		incorporation of ADP-		
			exogenous GTP (mM)	exogenous ATP (mM)	ribose (fmol/mg membrane protein) into polypeptides of	M_r	
						110 000	47 000 42 000
(i)	f	-	0.15	1.5	36	42	42
(i)	f	+	0.15	1.5	34	69	74
(i)	s	-	0.15	1.5	24	42	42
(i)	s	+	0.15	1.5	26	23	44
(ii)	f	-	0.15	1.5	35	42	42
(ii)	f	+	0.15	1.5	29	54	54
(ii)	f	+	0	0	36	42	42
(ii)	f	-	0.3	0	33	42	42
(ii)	f	+	0.3	0	39	59	59
(ii)* ¹	f	+	0.15	1.5	78	51	45
(ii)* ²	f	+	0.15	1.5	42	50	62

ADP-ribosylation by cholera toxin in the presence of ATP, but not GTP, was not tested as commercial preparations of ATP are contaminated with GTP (Kimura *et al.*, 1976).

was attempted to show that the substrates of cholera toxin were also in this part of the cell. Plasma membranes were purified from rat liver by the method of Pilkis et al. (1974), but cholera toxin-specific ADP-ribosylation of polypeptides could not be demonstrated in this preparation. This was probably because the system of inhibitors, buffers and incubation conditions which was used in this work was not designed for use with purified plasma membranes (Tait, 1980; Tait et al., 1980). But Doberska et al. (1980) used a different system to demonstrate ADP-ribosylation of a polypeptide of $M_r=42\ 500$ by cholera toxin in plasma membranes from rat liver.

4.2.6 Fractionation of radiolabelled proteins from rat liver membranes

Evidence suggested that cholera toxin ADP-ribosylates a GTPase which regulates adenylate cyclase (see sections 1.3.3.2 and 4.2.5). To test if the substrates of cholera toxin bound guanyl nucleotides, rat liver membranes were incubated with one or more of $[8-^3H]p(NH)ppG$, $[adenylate-^{32}P]NAD^+$ and cholera toxin. After washing the membranes, proteins were solubilized using detergent. The proteins were then fractionated in an attempt to show that both labels were attached to the same molecule. Fractionation techniques had to be mild and near to physiological pH to maximize the possibility that $p(NH)ppG$ would remain bound to GTPases despite the non-covalent nature of the bond.

4.2.6.1 Fractionation by gel-permeation chromatography

The Sephacryl column effectively fractionated proteins which had been solubilized from rat liver membranes (see section 2.10 and fig. 4.10(a)). This was confirmed by the fact that different fractions contained different polypeptides when they were analysed by SDS-PAGE (see section 2.8).

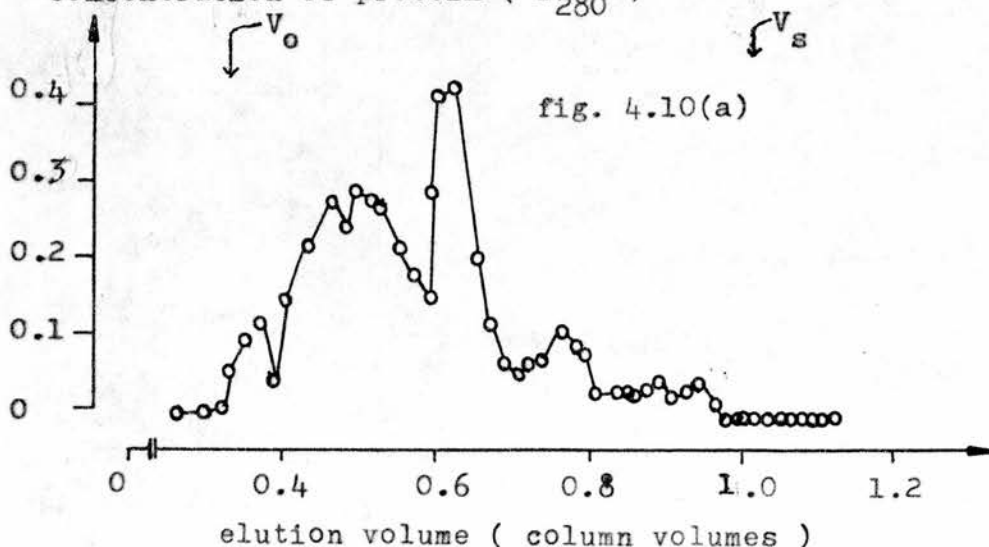
Solubilized proteins were firstly chromatographed on the Sephacryl column, then the fractions which made up the radio-

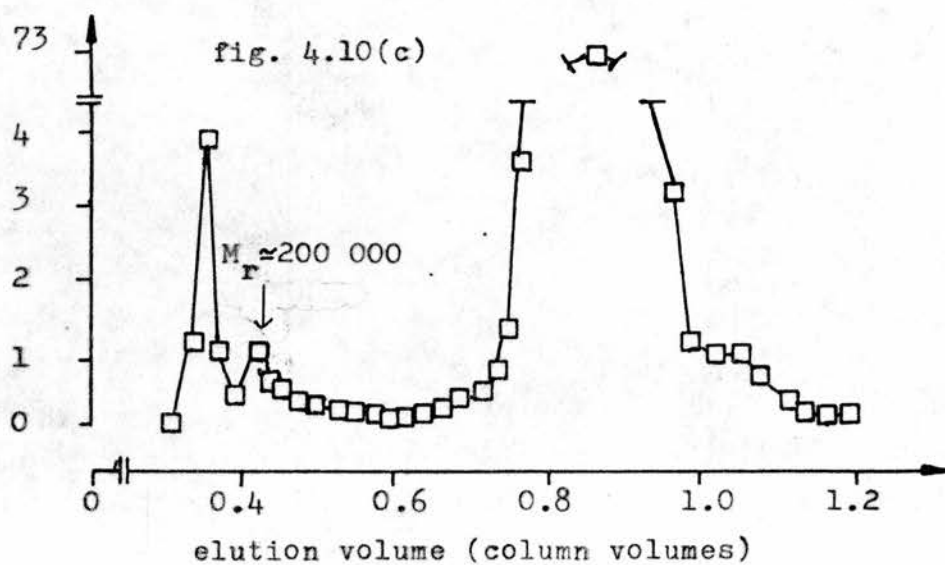
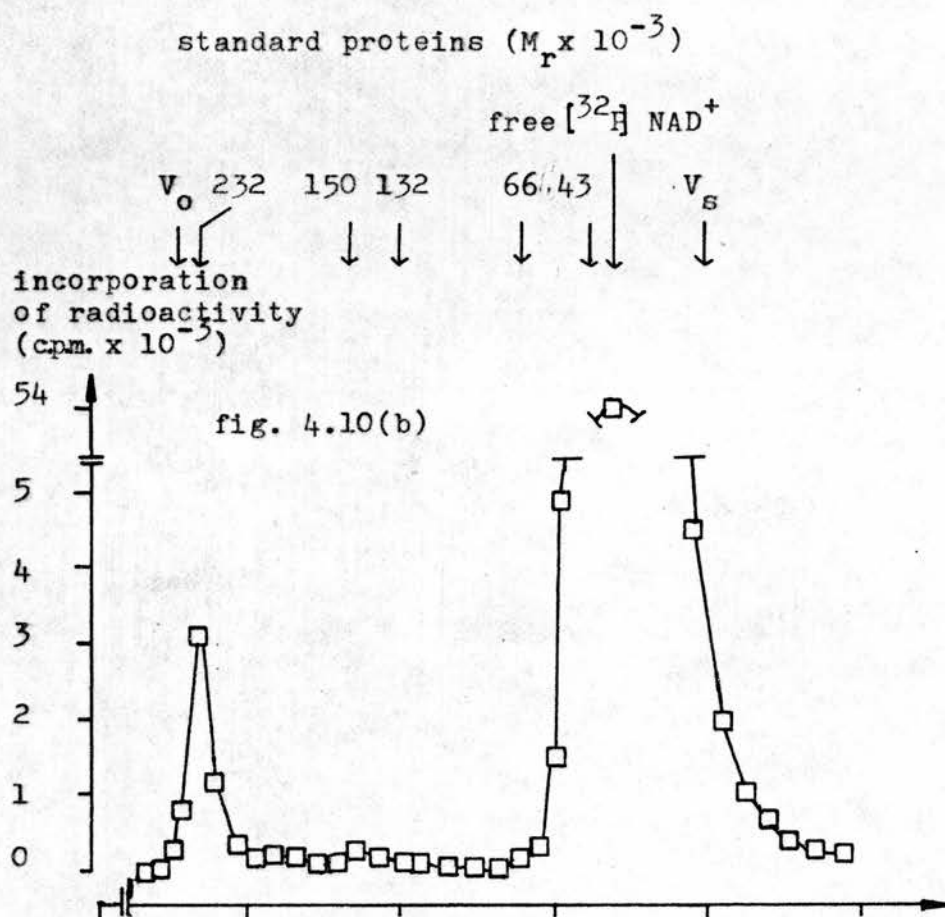
Fig. 4.10

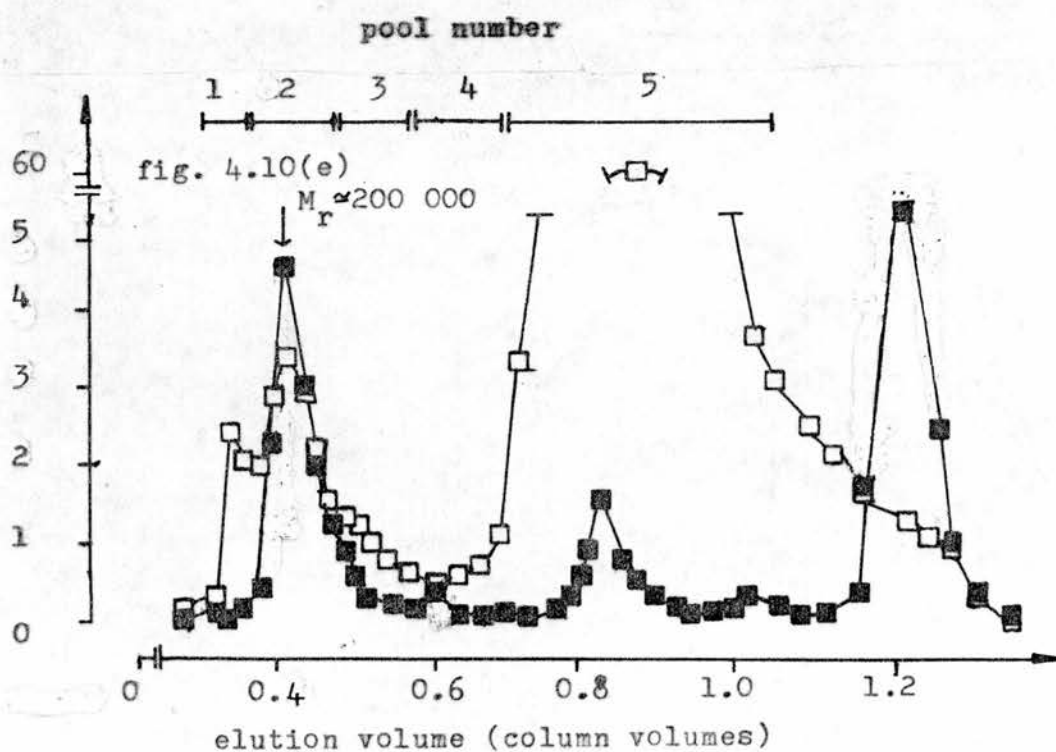
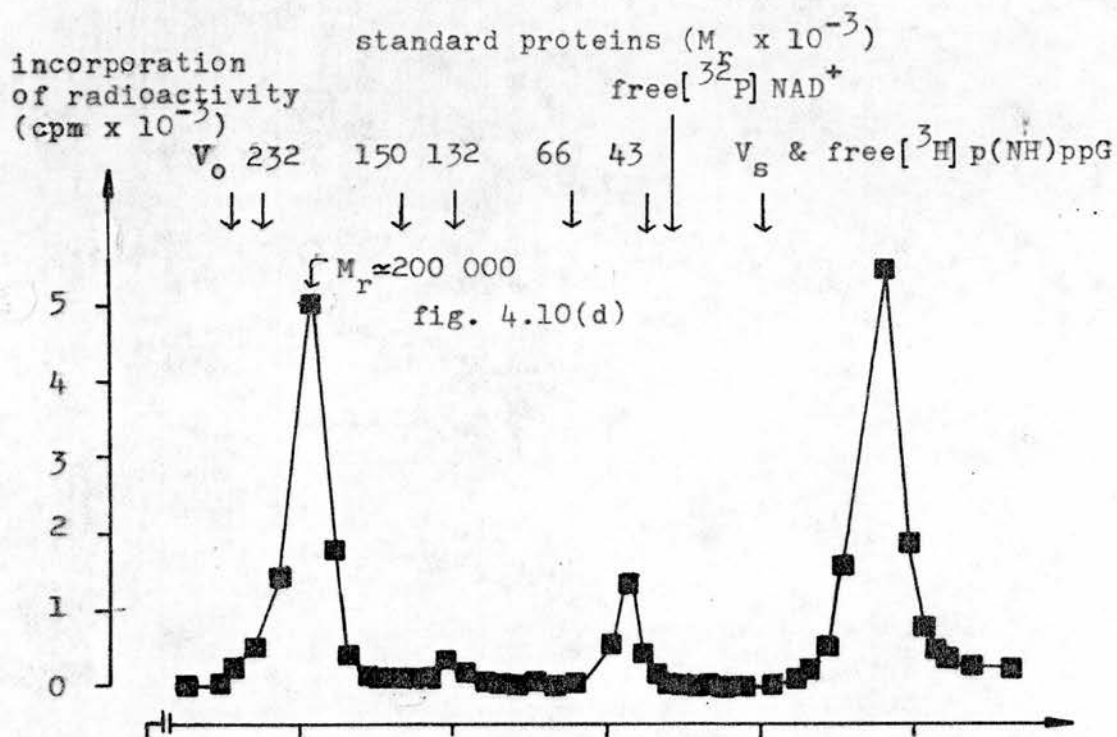
Gel-permeation chromatography of proteins labelled with
[8-³H] p(NH)ppG and [adenylate-³²P] NAD⁺

Rat liver membranes were incubated with one or more of [8-³H] p(NH)ppG, [adenylate-³²P] NAD⁺ and cholera toxin, washed three times and then solubilized by method (b) in section 4.1.4. When it was used, p(NH)ppG replaced GTP in the incubation of membranes with cholera toxin. [8-³H] p(NH)ppG was added to 10 μ M at 8.3Ci/mmol. Samples containing about 2mg protein were chromatographed using Sephacryl S-200 as described in section 2.10. Experimental details are given in section 2.6. The column was eluted at about 10cm³/h and fractions containing about 1.2cm³ were collected. The radioactivity in alternate fractions was measured and the values were corrected for cross-over of counts between the two isotopes (section 2.3). The background levels of radioactivity have been subtracted. All values are single determinations only, but similar results were obtained when the experiments were repeated. (a) Elution profile of proteins as determined by E₂₈₀. (b) Membranes pretreated with [adenylate-³²P] NAD⁺. (c) Membranes pretreated with [adenylate-³²P] NAD⁺ and cholera toxin. (d) Membranes pretreated with [8-³H] p(NH)ppG. (e) Membranes pretreated with [adenylate-³²P] NAD⁺, [8-³H] p(NH)ppG and cholera toxin. \square ³²P, \blacksquare ³Hx10⁻¹. V₀=exclusion volume as determined using blue dextran and V_s=elution position of 5-N dinitrophenyl lysine. [adenylate-³²P] NAD⁺ and [8-³H] p(NH)ppG were also chromatographed, eluting at the positions which are shown.

concentration of protein (E₂₈₀)







active peaks were pooled and fractionated on the Ultrogel column which resolved larger molecules (see section 2.10).

Pretreatment of membranes with γ -adenylate- ^{32}P /NAD⁺ in the absence of cholera toxin resulted in the labelling of material which eluted at the exclusion volume from both the Sephacryl column and the Ultrogel column (figs 4.10(b) and 4.11(a)). This high M_r material probably contained ADP-ribosylated nucleoprotein complexes (see Ueda *et al.*, 1979). When the protein in these fractions was precipitated with trichloroacetic acid, no radiolabelled polypeptides could be detected by SDS-PAGE and autoradiography (fig. 4.12), suggesting that a heterogeneous group of proteins was labelled and that each molecule was not sufficiently radioactive to be detectable by autoradiography. There was also some γ - ^3H /p(NH)ppG associated with this high M_r material (figs 4.10(d) and 4.11(a)), suggesting that it probably also contained molecules which were incompletely solubilized.

If membranes were pretreated with cholera toxin and γ -adenylate- ^{32}P /NAD⁺, a single toxin-specific peak eluted from the Sephacryl column with an apparent $M_r \approx 200\,000$ ($K_D = 0.11$) (fig. 4.10(c)). Although this was close to the exclusion volume for the column, these molecules eluted after an endogenously labelled peak, and after catalase. Therefore this estimate of M_r seems fairly reliable.

When membranes were preincubated with γ - ^3H /p(NH)ppG, the label eluted as several peaks (fig. 4.10(d)). One of the major species had an apparent $M_r \approx 200\,000$. The other large peak eluted after 5-N dinitrophenyl lysine, presumably because it interacted with the Sephacryl.

Pretreatment of membranes with γ - ^3H /p(NH)ppG, γ -adenylate- ^{32}P /NAD⁺ and cholera toxin produced all of the peaks that are described above (fig. 4.10(e)). The fractions containing high levels of ^{32}P were treated with trichloroacetic acid

Fig. 4.11

Gel-permeation chromatography of proteins labelled with γ - ^3H p(NH)ppG and γ -adenylate- ^{32}P /NAD $^+$

Radiolabelled proteins were prepared as described in the legend to fig. 4.10. The samples were chromatographed on an Ultrogel column as described in section 2.10. The column was eluted at about $18\text{cm}^3/\text{h}$ and fractions containing about 5cm^3 were collected. The radioactivity in alternate fractions was measured and values were corrected for cross-over of counts between the two isotopes (section 2.3). The background levels of radioactivity were subtracted. All values are single determinations, and similar results were obtained when the experiment was repeated.

(a) Pool 1 from fig. 4.10 (e)

(b) Pool 2 from fig. 4.10 (e)

□ ^{32}P , ■ $^3\text{H} \times 10^{-1}$. V_o = exclusion volume as determined using blue dextran and V_e = elution position of 5-N dinitrophenyl lysine. γ -adenylate- ^{32}P /NAD $^+$ and γ - ^3H p(NH)ppG were also chromatographed and their elution positions are shown.

standard proteins ($M_r \times 10^{-3}$)

incorporation of radioactivity ($\text{cpm} \times 10^{-1}$)

V_0 232 160 147 66 & 64 free [^{32}P] NAD^+ ; V_s & free [^3H] p(NH)ppG

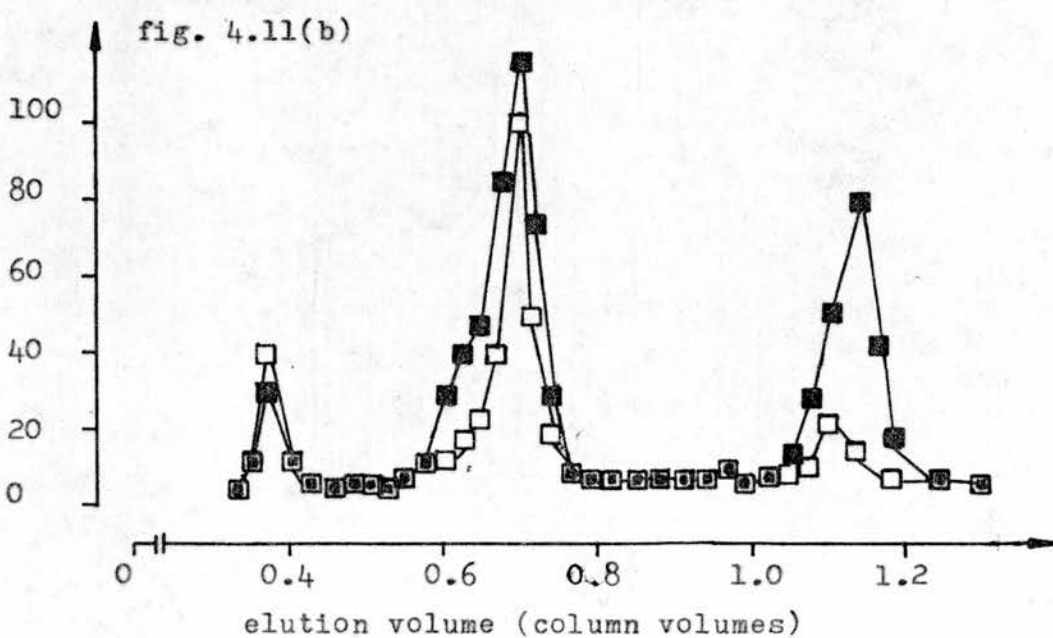
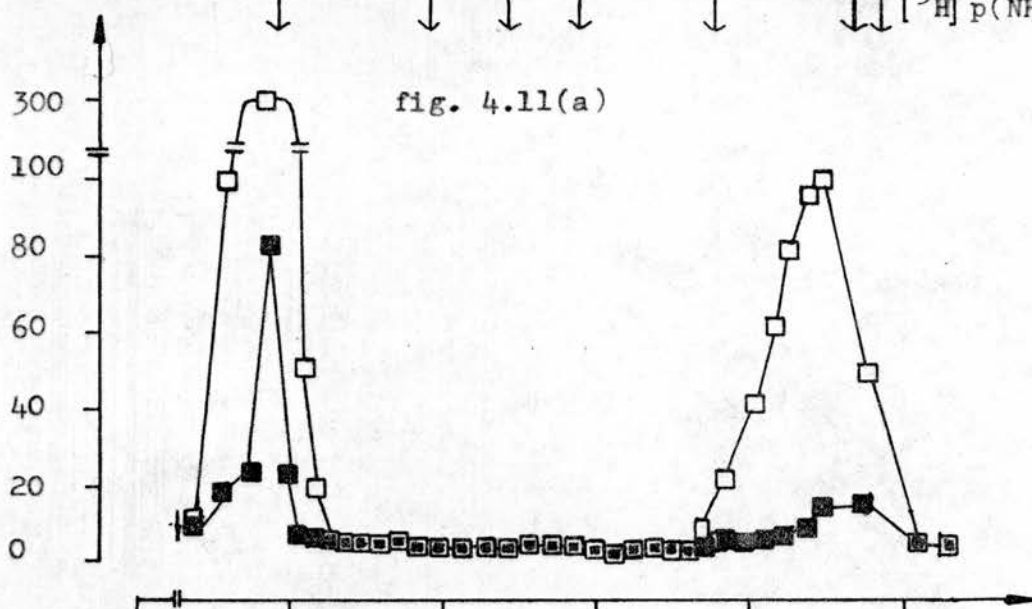


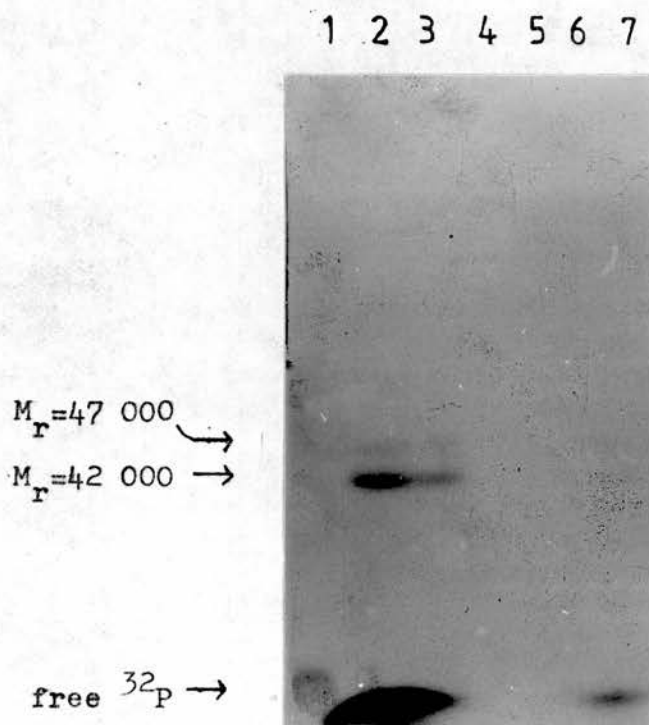
Fig. 4.12

SDS-PAGE of ADP-ribosylated polypeptides from rat liver membrane proteins which were solubilized and then fractionated by gel-permeation chromatography

Radiolabelled proteins were prepared as described in the legend to fig. 4.10 (e). The proteins were precipitated (see section 4.1.6) and then analysed by SDS-PAGE and autoradiography (sections 2.8 and 2.9). The final concentration of acrylamide in the separating gel was 8% (w/v). Similar results were obtained when the experiment was repeated.

Lane 1 control membranes (not treated with cholera toxin).	}	see section 2.6 and legend to fig. 4.10 (e).
Lane 2 membranes treated with cholera toxin. (This material was used as the sample for gel-permeation chromatography.)		

Pools from fig. 4.10(e). Lane 3: pool 2. Lane 4: pool 3. Lane 5: pool 4. Lane 6: pool 5. Lane 7: pool 1.



to precipitate the proteins which were then analysed by SDS-PAGE and autoradiography. The peak of $M_r \approx 200\ 000$ contained both principal substrates of cholera toxin (M_r of 42 000 and 47 000) as the only labelled polypeptides (fig. 4.12). No polypeptides containing ^{32}P could be detected in the other fractions (fig. 4.12). This technique would not detect proteins labelled with $[\gamma\text{-}^3\text{H}]\text{p}(\text{NH})\text{ppG}$ as the ligand was not covalently attached, therefore it would be liberated by the precipitation and SDS-PAGE procedures. Various other techniques were tried to fractionate the peaks from the Sephacryl column (see the other parts of section 4.2.6).

The fractions which made up each of pool 1 and pool 2 from the Sephacryl column (see fig. 4.10(e),) were analysed on the Ultrogel AcA34 gel-permeation column. After this treatment, both pools contained radioactive material of very high M_r (which was predominantly incompletely solubilized material) and low M_r species which were detached radiolabels (fig. 4.11).

This showed pool 1 contained no other significant radioactive species. The peak of apparent $M_r \approx 200\ 000$ in the Sephacryl system eluted with an apparent $M_r \approx 160\ 000$ from Ultrogel. These values agreed quite well. Both radiolabels again fractionated together.

Free $[\gamma\text{-}^3\text{H}]\text{p}(\text{NH})\text{ppG}$ eluted from both gel-permeation columns at the same position as 5-N dinitrophenyl lysine. This was to be expected for material of low M_r . But free $[\text{adenylate-}^{32}\text{P}]\text{NAD}^+$ eluted from both columns with an anomalously high apparent M_r , presumably because of repulsion effects between it and the bed material of the columns or some other part of the chromatography system, or because it became part of large aggregations. Membranes were washed three times before solubilization but, although this procedure effectively removed the free $\text{p}(\text{NH})\text{ppG}$, it apparently failed to remove all

of the free NAD^+ (fig. 4.10). NAD^+ had probably become sequestered in membraneous vesicles (e.g. nuclei) which were washed free of external radiolabel and then subsequently lysed, by the solubilization procedure, to release free NAD^+ . Alternatively NAD^+ may have bound to membranes and then been released by the detergent.

It is a coincidence that a species which bound p(NH)ppG eluted at a similar position to free NAD^+ (fig. 4.10(a)). But this was a genuine phenomenon as the peak was seen when membranes were preincubated with p(NH)ppG in the absence of NAD^+ (fig. 4.10(c)).

4.2.6.2 Ion-exchange chromatography of proteins solubilized from rat liver membranes

Proteins were solubilized from rat liver membranes and analysed using two-dimensional electrophoresis (see section 4.1.7). Proteins were separated according to isoelectric point by non-denaturing electrophoresis in polyacrylamide containing the detergent Lubrol PX. After this "first dimension", polypeptides were fractionated by SDS-PAGE. Almost all of the polypeptides had isoelectric points in the range $\text{pH} 5-5.4$ (fig. 4.13). Some polypeptides failed to enter the gel in the first dimension probably because they were not completely solubilized.

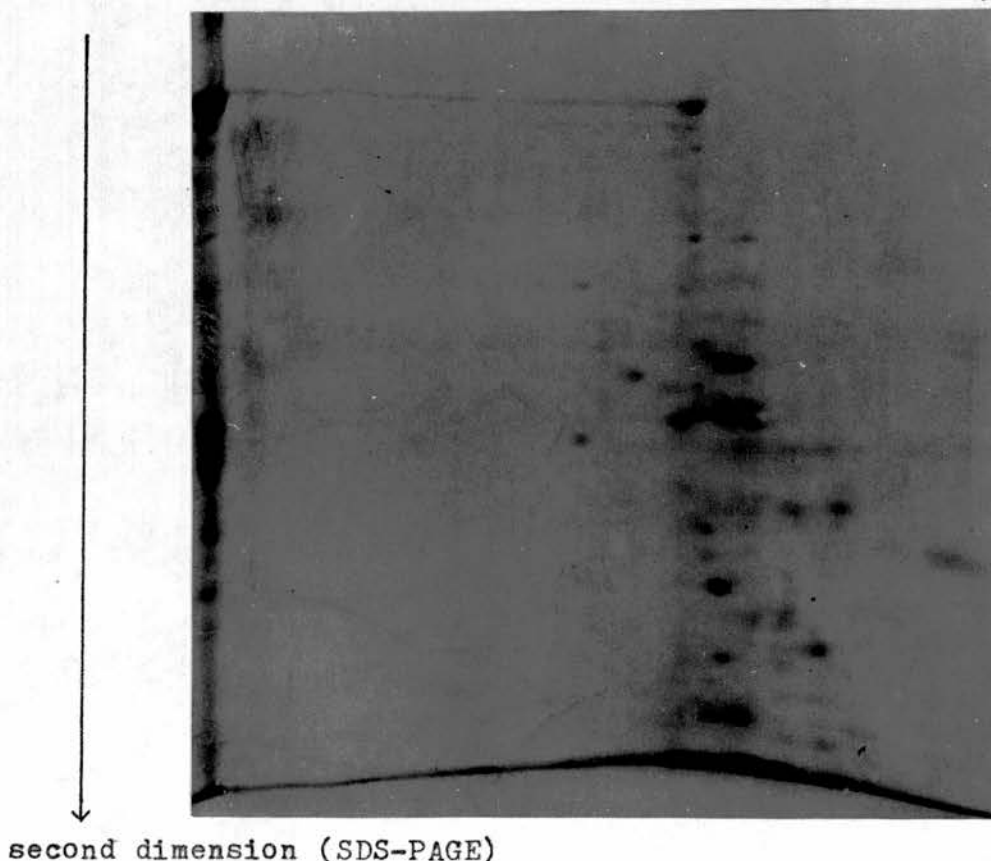
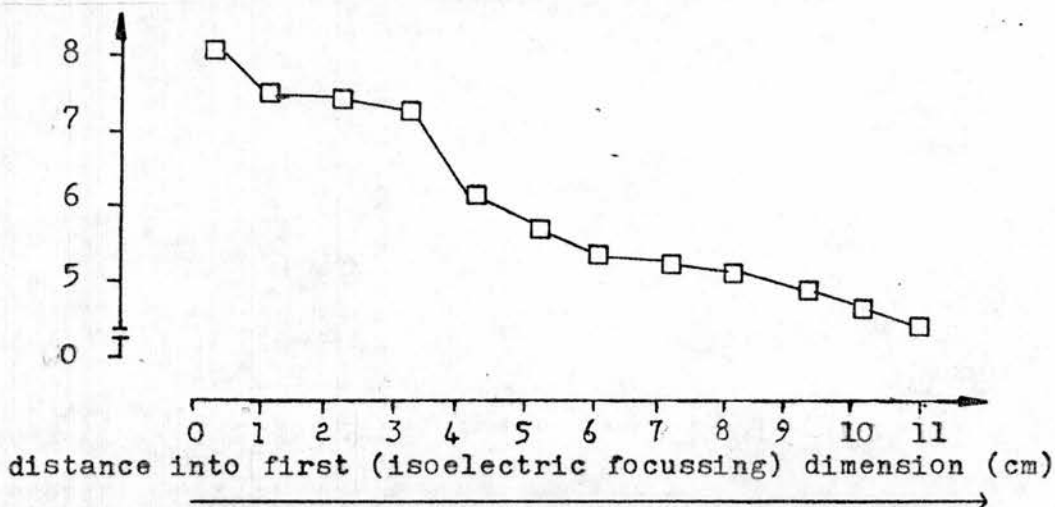
The proteins were probably covered by Lubrol PX or lipids from the membranes causing the complexes to have similar isoelectric points. All of the proteins would have hydrophobic regions on their surfaces because of their location in, or on, membranes. This would limit the variation which was possible in the chemical nature of the surface of the proteins. A narrow range of isoelectric points has also been observed for proteins solubilized from the membranes of bovine chromaffin granules (R. Sutton, personal communication).

Fig. 4.13

Two-dimensional electrophoresis of polypeptides from rat liver membranes.

Rat liver membranes were prepared as described in section 2.1.2. Proteins were solubilized from the membranes by method (b) in section 4.1.4 and then analysed by two-dimensional PAGE (see section 4.1.7). The sample was applied to the high end of the pH gradient. Similar results were obtained when the experiment was repeated.

pH in the
first dimension



Most of the membrane proteins would therefore be expected to have a negative charge at physiological pH values therefore it was attempted to fractionate the proteins on a cation exchange resin. The details of these experiments are given in the legends to figs 4.14 and 4.15. The basis of the investigation was as follows.

Membranes were incubated with cholera toxin, γ -adenylate- ^{32}P NAD $^{+}$ and γ - ^3H -p(NH)ppG, and then washed. This procedure was shown to remove most of the free NAD $^{+}$ and effectively all of the free p(NH)ppG (see section 4.2.6.1). Proteins were then solubilized with the non-ionic detergent Lubrol PX and then chromatographed on a Whatman DE-52 ion-exchange column. After fractionation, the proteins were precipitated with trichloroacetic acid and then radioactive polypeptides were separated by SDS-PAGE, and identified by autoradiography.

Most of the radiolabelled material failed to bind to the column (fig. 4.14), but both substrates of cholera toxin (the polypeptides of $M_r=42\ 000$ and $47\ 000$) could be detected in the washes with 100mM NaCl and 400mM NaCl (fig. 4.15). This roughly coincided with a peak in the elution of γ - ^3H -p(NH)ppG; an observation which is consistent with cholera toxin modifying a protein which binds guanyl nucleotides. It is unclear why the protein eluted at two different concentrations of NaCl (see section 4.3.5). There appeared to be only small amounts of the substrates of cholera toxin occurring in the unbound material (fig. 4.15). There was not a significant peak of ^{32}P in the elution profile. This correlates with the large amounts of unbound ^{32}P found in the solubilized preparations on gel-permeation chromatography (fig. 4.10(b)), but the small amount of labelling made it difficult to clearly demonstrate that the radiolabels eluted together. Ion-exchange chromatography could form a useful part of a purification procedure for the substrates of cholera toxin.

Fig. 4.14

Ion-exchange chromatography of proteins solubilized from rat liver membranes

Rat liver membranes were incubated with $5\mu\text{M}$ γ -adenylate $^{32}\text{P}/\text{NAD}^+$ and cholera toxin (see section 2.6). $10\mu\text{M}$ γ - $^3\text{H}/\text{p}(\text{NH})\text{ppG}$ ($8.3\text{Ci}/\text{mmol}$) replaced GTP in the incubation. Membranes were washed three times and then solubilized by method (b) in section 4.1.4. A sample containing 2.5mg protein was chromatographed on a column of Whatman DE-52 ion-exchange resin as described in section 4.1.8. The concentration of NaCl in the elution buffer was increased step-wise as shown. The values for the radioactivity in the fractions were corrected for cross-over of counts from the two isotopes (section 2.3). The background levels of radioactivity were subtracted. All values were only single determinations, but similar results were obtained when the experiment was repeated. Under the conditions of this experiment neither γ - $^3\text{H}/\text{p}(\text{NH})\text{ppG}$ nor γ -adenylate- $^{32}\text{P}/\text{NAD}^+$ bound to the ion-exchange column.

□ ^{32}P ■ ^3H NaCl

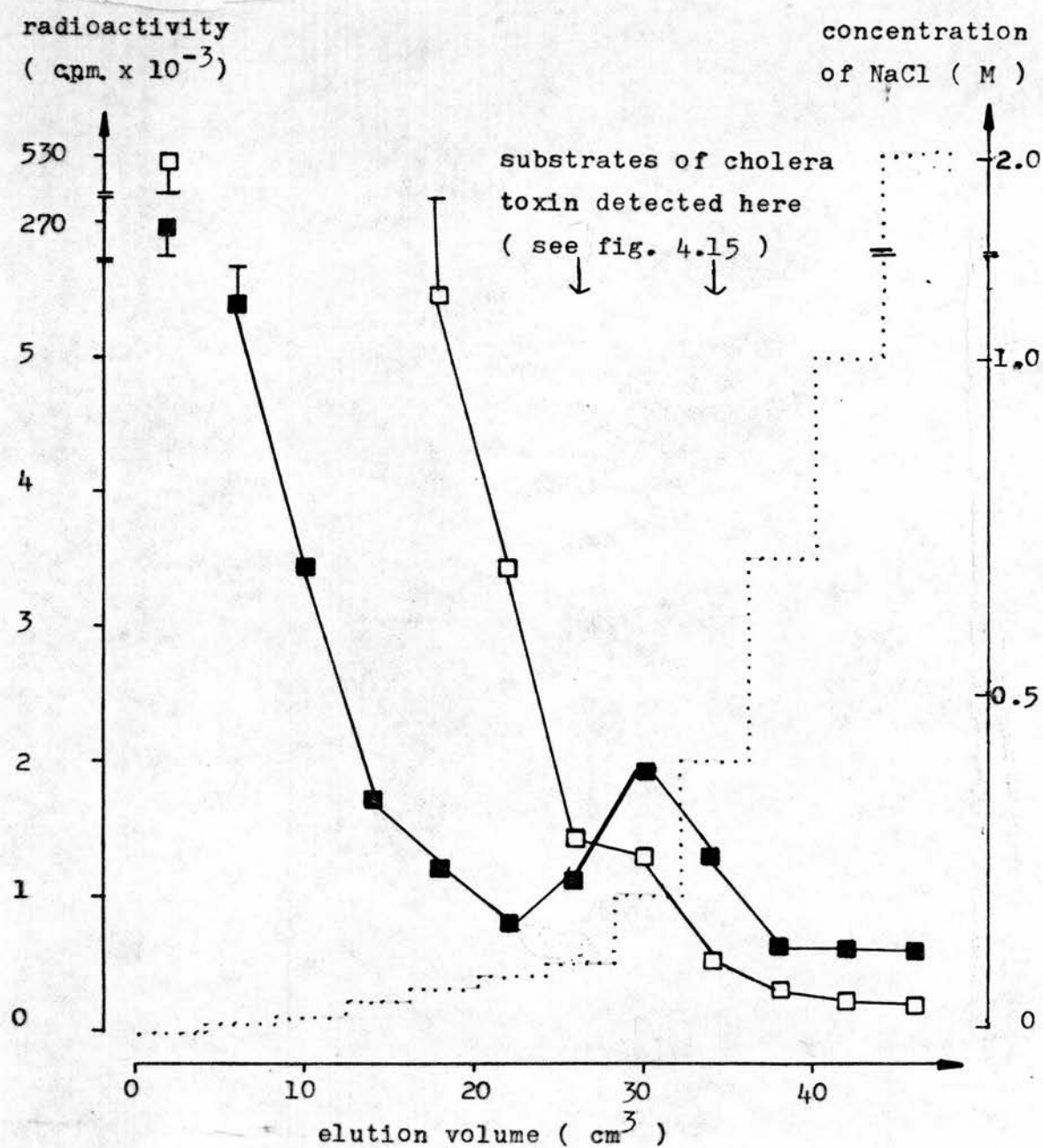


Fig. 4.15

SDS-PAGE of ADP-ribosylated polypeptides from rat liver membrane proteins which were solubilized and then fractionated by ion-exchange chromatography

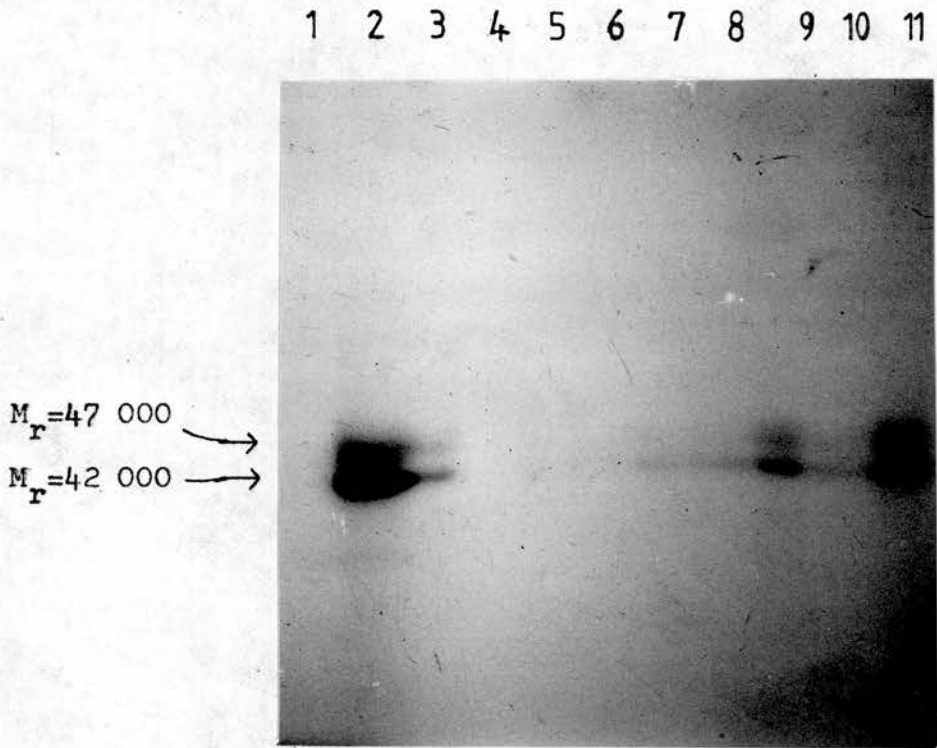
Radiolabelled polypeptides were prepared as described in the legend to fig. 4.14. The proteins were precipitated (see section 4.1.6) and then analysed by SDS-PAGE and autoradiography (sections 2.8 and 2.9). The final concentration of acrylamide in the separating gel was 8%(w/v). Similar results were obtained when the experiment was repeated.

Lane 1 control membranes (not treated with cholera toxin).	}	see section 2.6 and legend to fig. 4.14.
Lane 2 membranes treated with cholera toxin. (This material was used as the sample for ion-exchange chromatography.)		

The other lanes contained proteins which had eluted with different concentrations of NaCl as shown in fig. 4.14.

Lane 3 : 0mM NaCl
Lane 4 : 10mM NaCl
Lane 5 : 20mM NaCl
Lane 6 : 40mM NaCl
Lane 7 : 60mM NaCl
Lane 8 : 80mM NaCl
Lane 9 : 100mM NaCl
Lane 10 : 200mM NaCl
Lane 11 : 400mM NaCl

Fig. 4.15



Fractions from the column were also analysed by SDS-PAGE followed by staining for protein (see section 2.8). This showed that ion-exchange chromatography was effectively fractionating proteins. Therefore the co-elution of the ^{32}P -radiolabelled polypeptides and $[8\text{-}^3\text{H}]\text{p}(\text{NH})\text{ppG}$ was a genuine result.

The two principal substrates of cholera toxin fractionated together on gel-permeation chromatography (fig. 4.12) and on ion-exchange chromatography (fig. 4.15). This is strong evidence showing that both polypeptides occurred in the same protein.

4.2.6.3 Fractionation of proteins using ammonium sulphate

Membranes from rat liver were incubated with cholera toxin and $[\text{adenylate-}^{32}\text{P}]\text{NAD}^+$ (section 2.6), washed and then solubilized by method (b) in section 4.1.4. The concentration of ammonium sulphate in the preparation of solubilized proteins was increased step-wise (see section 4.1.8) but these attempts to purify the substrates of cholera toxin failed for two reasons.

- (a) Ammonium ions induced detachment of the labelling ADP-ribose group. This has also been found for the ADP-ribose-histidine linkage in histones (Ueda *et al.*, 1979; Hilz, 1981).
- (b) SDS-PAGE (section 2.8) showed that the different polypeptides in the preparation all had similar solubilities in ammonium sulphate. This was probably because the proteins were surrounded by a coat of detergent. This observation is consistent with the result that the polypeptides all had similar isoelectric points (fig. 4.13).

4.2.6.4 Non-denaturing electrophoresis of radiolabelled proteins

The pools of fractions from the Sephacryl gel-permeation column (see fig. 4.10(e)) were analysed using non-denaturing electrophoresis in four different gel systems (table 4.14). The final (w/v) concentration of acrylamide in the stacking gels was 2.5% and in the separating gels was 5%. Gels were sliced, digested and then counted for radioactivity (see section 2.8.2). Very little radioactivity entered the gels. Proteins were solubilized from rat liver membranes by method (b) in section 4.1.4, and then analysed in the same gel systems. Staining with Coomassie Brilliant Blue (section 2.8.1) showed that most of the protein failed to enter the gels.

Presumably the proteins were not charged at the pHs of the electrophoreses as they were covered with a shell of non-ionic detergent. This probably explains why they failed to enter the gels.

4.2.7 An investigation to test if the groups that are ADP-ribosylated by cholera toxin are in the binding sites for guanyl nucleotides on the protein which regulates adenylate cyclase

Cholera toxin activates adenylate cyclase by modifying a GTPase which is a regulatory component of the enzyme (see sections 1.3.3 and 4.3.5). When stimulated by a hydrolysis-resistant analogue of GTP (p(NH)ppG), adenylate cyclase could not be further stimulated by cholera toxin (table 4.11, Flores & Sharp, 1975). It is therefore possible that cholera toxin could modify groups which are involved in the binding site for guanyl nucleotides on the regulatory protein.

To test this hypothesis, membranes were incubated with 1mM p(NH)ppG for 20min, washed and then treated with cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$. The membranes were washed and then

Table 4.14

Types of non-denaturing electrophoresis which were used in an attempt to fractionate proteins solubilized from rat liver membranes

Experimental details are given in the text (section 4.2.6.4).

<u>detergent used in the gel</u>	<u>pH of gel buffer</u>	<u>reference</u>
Lubrol PX to 0.1% (w/v)	7.5	Newby <u>et al.</u> , [*] (1978)
Lubrol PX to 0.1% (w/v) and SDS to 0.03% (w/v)	7.5	Newby & Chrambach (1979)
None	8.9	Davis (1964)
Lubrol PX to 0.1% (w/v)	8.9	*

*The gel system was the same as that of Davis (1964) with the addition of Lubrol PX in the gel and in the electrode buffer.

assayed for the amount of ADP-ribosylation which had occurred. In control experiments, the sequence of the two incubations was reversed. Pretreatment of the membranes with p(NH)ppG failed to block ADP-ribosylation by cholera toxin (table 4.15). This result was consistent with the observed requirement for GTP in the action of cholera toxin (tables 4.2 and 4.13). But, as exchange of guanyl nucleotides occurs at the regulatory site, GTP could dissociate to allow access for cholera toxin. After preincubation of rat liver membranes with γ - ^3H -p(NH)ppG, washing and solubilization, no dissociation of ^3H was detectable by gel-permeation chromatography as there was no free ^3H and no trailing of the peaks of ^3H -labelled protein (fig. 4.10(d)). The high affinity of p(NH)ppG for the regulatory component of adenylate cyclase was also indicated by the observation that stimulation of the enzyme by p(NH)ppG was stable to washing (fig. 2.8). Preincubation with 10 μM p(NH)ppG caused maximal activation of adenylate cyclase in rat liver membranes (fig. 2.8). Similar concentrations of the analogue probably saturated the sites to which it bound in the same system (fig. 4.16). This showed that p(NH)ppG remained bound to all of its binding sites on the regulatory protein during the experiment which is described above (see table 4.15). These observations are in agreement with the results of Rodbell *et al.* (1975) who found that p(NH)ppG bound very tightly to plasma membranes from rat liver.

Table 4.15

ADP-ribosylation of polypeptides by cholera toxin in the presence of p(NH)ppG

Rat liver membranes were incubated in the absence of GTP, but with 1mM p(NH)ppG, for 20min (see section 2.1), washed and then treated with cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$ (see section 2.6). In the control experiment, the sequence of the two incubations was reversed. After washing, ADP-ribosylation was measured as described in section 2.9.3. The values are single determinations only, but similar results were obtained when the experiments were repeated.

<u>treatment of membranes</u>	<u>incorporation of ADP-ribose (fmol/mg membrane protein) into polypeptides of M_r</u>	
	<u>47 000</u>	<u>42 000</u>
incubated with p(NH)ppG		
in the absence of cholera toxin	< 2	< 2
incubated with cholera toxin and then p(NH)ppG	60	66
incubated with p(NH)ppG and then cholera toxin	51	63

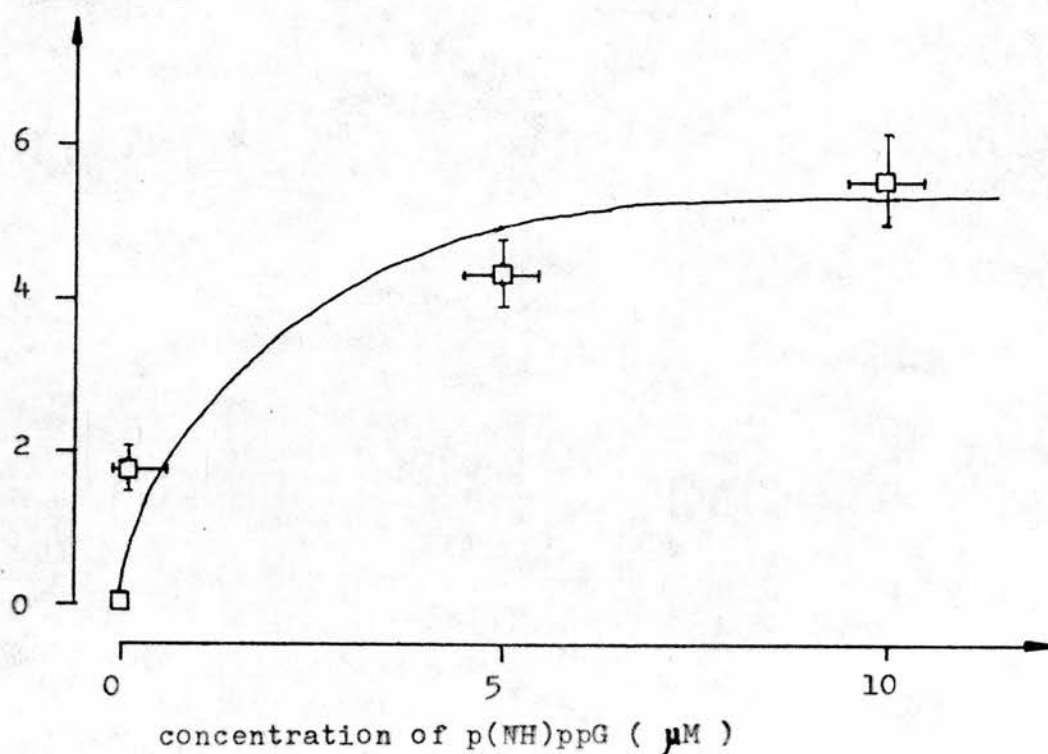
0.5mg membrane protein was used in each ADP-ribosylation assay.

Fig. 4.16

Binding of p(NH)ppG to membrane proteins

Rat liver membranes were incubated in the absence of GTP, but in the presence of various concentrations of $[8-^3\text{H}]$ p(NH)ppG (at a specific activity of 8.3Ci/mmol). The membranes were washed three times and then the amount of bound radiolabel was counted (see sections 2.1.2, 2.1.4 and 2.3). The values were single determinations only, but similar results were obtained when the experiment was repeated. The estimates of the errors in the data points are shown in the figure. About 2mg membrane protein was used for each data point.

$[8-^3\text{H}]$ p(NH)ppG
bound (cpm, $\times 10^{-5}/\text{mg}$
membrane protein)



4.3 DISCUSSION

4.3.1 Labelling of membrane proteins by cholera toxin and radioactive NAD⁺

The levels of endogenous incorporation of radiolabel from NAD⁺ were very low in comparison to those reported by other workers using rat liver (see fig. 4.1 and Doberska et al., 1980). This was probably due to the use of a potent combination of inhibitors of NAD⁺-degrading enzymes in the current work. The low usage of NAD⁺ for labelling by endogenous enzymes correlates with the low concentration of NAD⁺ that was required for maximal activation of adenylate cyclase by cholera toxin and maximal labelling of membrane proteins by cholera toxin (figs 4.3 and 4.4).

Other workers studying the action of cholera toxin in rat liver or related systems have disagreed on the identity of the substrates of cholera toxin (table 4.16).

In the current work the predominant substrates of cholera toxin had M_r 's of 47 000 and 42 000. The polypeptide of $M_r=42$ 000 was the principal receptor of radiolabel. Molecules of M_r 42 000 and 47 000 were ADP-ribosylated by cholera toxin in human fibroblasts (Watkins et al., 1981). The 42 000 M_r -substrate of cholera toxin could be structurally related to the 47 000 M_r -substrate (see section 5.2). Proteolysis could then generate the smaller molecule from the larger one. This could explain why a single substrate was detected in plasma membranes which were prepared from rat liver (table 4.16). Proteolysis could have occurred during purification of the membranes. This hypothesis is consistent with the observation that the smaller substrate always predominates in systems where two molecules are labelled (figs 4.2 and 4.4; Hudson & Johnson, 1980). Different workers may detect different substrates of cholera toxin because they used different buffers, toxin or experimental

Table 4.16

The principal substrates of cholera toxin in rat liver and related systems

Membranes were incubated with cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$. The M_r 's of the substrates of cholera toxin were determined by SDS-PAGE and autoradiography.

<u>M_r's of substrates</u>	<u>notes</u>	<u>reference</u>
42 500	used purified plasma membranes	Doberska <u>et al.</u> (1980)
45 000 52 000	used HTC4 rat hepatoma cells	Hudson and Johnson (1980)
	55 000* used RL-PR-C hepatocytes	Beckner and Blecher (1980)
42 000 47 000	56 000*	current work (figs 4.1 and 4.2)

*In both systems this molecule was also labelled to a lesser extent in the absence of cholera toxin.

systems (especially inhibitors of enzymes which degrade NAD^+ or inhibitors of proteolytic enzymes).

In the presence of GTP, cholera toxin inhibited cAMP-dependant protein kinase (Walaas et al., 1981). In the same preparation of sarcolemmal membranes, cholera toxin ADP-ribosylated a polypeptide of $M_r=56\ 000$. The regulatory subunit of protein kinase has a $M_r=56\ 000$ (Walaas et al., 1981). The cholera toxin-substrate of $M_r=56\ 000$ which was detected in the current work (figs 4.1 and 4.2) may be the regulatory subunit of protein kinase.

4.3.2 Activation of adenylate cyclase by cholera toxin

The stimulation of adenylate cyclase by cholera toxin was dependant upon NAD^+ (fig. 4.3). $1\mu\text{M}$ NAD^+ produced maximal activation of the enzyme by cholera toxin whereas about $10\mu\text{M}$ NAD^+ was required for maximal ADP-ribosylation by cholera toxin (figs 4.3 and 4.4). The NADase activity of preparations of cholera toxin had a K_m of 4mM (Moss et al., 1976b). Therefore these results showed that the preparation of cholera toxin that was used in the current work had a higher specificity for ADP-ribosyl transferase activity than Moss's preparation had for NADase activity. The need for NAD^+ in the action of cholera toxin is well-known, therefore compounds which are related to NAD^+ were not tested for the ability to support the action of the toxin. This has been extensively studied in other systems (Gill, 1975).

The requirement for GTP in the activation of rat liver adenylate cyclase by cholera toxin (table 4.2) is in agreement with the results of Lin et al. (1978). Doberska et al. (1980) confirmed the observation that ADP-ribosylation of rat liver proteins by cholera toxin was also dependent upon GTP (table 4.13).

In the current work cytosol was not required for the activation

of adenylate cyclase nor for ADP-ribosylation by cholera toxin (section 4.2.2). These observations were in agreement with the results of Tait (1980) and Doberska *et al.* (1980), but not with those of Flores *et al.* (1976). A rat liver system was used in all cases, and the cytosol was always diluted in homogenisation buffer. In the current work, adenylate cyclase activity was higher in the presence of cytosol, but basal and cholera toxin-stimulated activities were increased to similar extents (table 4.3). Cytosol may contain factors which elevate the activity of adenylate cyclase (Katz *et al.* 1978). Plasma membranes contain proteins which are required for the action of cholera toxin (Pinkett & Anderson, 1982; Schleifer *et al.*, 1982). An apparent requirement for cytosol may have been seen because these factors were washed off the membranes during preparation.

4.3.3 ADP-ribosylation by cholera toxin

The results presented in the current work (section 4.2.3) showed that cholera toxin was almost certainly acting as an ADP-ribosyl transferase.

The high levels of nicotinamide that were required to block the action of cholera toxin (fig. 4.5), correlate with the difficulty in reversing the effects of cholera toxin (tables 4.4 and 4.5). Both observations suggest that the equilibrium of the reaction that is catalyzed by cholera toxin (fig. 1.7) is in favour of the ADP-ribosylated products.

There is strong evidence that cholera toxin transfers ADP-ribose rather than any other part of NAD^+ (see sections 1.3.3 and 4.2.3). Therefore nicotinamide was the only derivative of NAD^+ which was tested for its ability to inhibit cholera toxin. Inhibition of cholera toxin-specific radiolabelling by nicotinamide paralleled its effect on the activation of adenylate cyclase by cholera toxin (see fig. 4.5 and table

4.5). This was consistent with the hypothesis that the stimulation of adenylate cyclase by cholera toxin involves ADP-ribosylation (see section 1.3.3).

4.3.4 Interaction of cholera toxin with arginine, or arginine-like, residues

Arginine inhibited the activation of adenylate cyclase by cholera toxin in avian erythrocytes (Gill, 1979). The amino-acid had no detectable effect on the ADP-ribosylation of proteins by cholera toxin (Gill, 1979). Arginine methyl ester inhibited both the activation of adenylate cyclase and ADP-ribosylation by cholera toxin in the present work (fig. 4.6 and table 4.7). High levels of arginine methyl ester were required for inhibition, suggesting that it was competing with the natural substrate for the catalytic site of the toxin. High concentrations of the inhibitor may be required because the natural substrate is a modified arginine residue (see section 5.3.3) or because the chemical environment is important in determining the substrate specificity. The second hypothesis is supported by the observation that the ADP-ribosylation of artificial receptors was not directly related to the numbers of guanidino-groups which they contained (Mekalanos et al., 1979).

In rat hepatocytes arginine decreased the ADP-ribosylation of a substrate of $M_r=55\ 000$ by cholera toxin. Lysine had no effect (Beckner & Blecher, 1981). The effects of the amino-acids on the activation of adenylate cyclase by cholera toxin were not tested. In the current work arginine methyl ester or lysine methyl ester blocked ADP-ribosylation of cholera toxin-substrates of $M_r=42\ 000$ and $47\ 000$ (table 4.7). The radiolabelling of a molecule of $M_r=56\ 000$ by cholera toxin was too small to measure accurately (figs 4.1 and 4.2). Differences between the results of the current study and those of other workers may be because methyl esters are better models than free amino-acids for residues in proteins, or because in

the current work inhibitors were used at much higher concentrations than in other studies, or because of differences between the experimental systems. The effects of arginine methyl ester and related compounds on the activation of adenylate cyclase by cholera toxin paralleled the effects on ADP-ribosylation (tables 4.6 and 4.7). This was consistent with the hypothesis that the stimulation of adenylate cyclase by cholera toxin involves ADP-ribosylation (see section 1.3.3). Lysine methyl ester completely inhibited ADP-ribosylation by cholera toxin, but only partially blocked the activation of adenylate cyclase (tables 4.6 and 4.7). This apparent contradiction was probably because slight effects of cholera toxin were more easily detected in the adenylate cyclase assay than in the ADP-ribosylation assay as the former system produces a catalytically active species. The results of the current work correlate with the effects of the same compounds on the ability of preparations of cholera toxin to catalyse the release of nicotinamide from NAD^+ (Moss & Vaughan, 1977). The results in section 4.2.4 suggest that cholera toxin is interacting with arginine, or some arginine-like, residues on the polypeptides which it ADP-ribosylates.

4.3.5 Modification of the regulatory component of adenylate cyclase by cholera toxin

The results which are presented in the current work showed that cholera toxin modified the function of a protein which bound guanyl nucleotides and which regulated the activity of adenylate cyclase (sections 4.2.5 and 4.2.6). Cholera toxin also ADP-ribosylated this protein (see sections 4.2.5 and 4.2.6).

The regulatory component of adenylate cyclase has been purified from rabbit liver. It is made up of polypeptides of $M_r=52\ 000$, $45\ 000$ and $35\ 000$. The protein contained one molecule of each polypeptide giving it a $M_r=132\ 000$. Only the two heavier polypeptides were ADP-ribosylated by cholera toxin (Northup et al., 1980). The principal substrates of cholera toxin in

the current work (M_r 's about 42 000 and 47 000, see section 4.2.1) had similar sizes to the polypeptides in the regulatory component of adenylate cyclase from rabbit liver.

The role of each of the substrates of cholera toxin in the regulation of adenylate cyclase is unclear. Either one of the polypeptides has the ability to change the activity of the enzyme (Hanski et al., 1981; Kaslow et al., 1981b; Sternweis et al., 1981). The polypeptide of $M_r=35$ 000 from the regulatory protein is required before either of the substrates of cholera toxin has any effect (Hanski et al., 1981; Sternweis et al., 1981). The regulatory component of adenylate cyclase in avian erythrocytes lacks the polypeptide of $M_r=52$ 000 but it does have the ability to regulate the activity of the enzyme (Hanski et al., 1981; Sternweis et al., 1981). In other sources the regulatory protein contains a second substrate for cholera toxin (see fig. 4.1 in the current work; Johnson, 1982; Limberd, 1981). When such systems are compared with those which lack the largest polypeptide, they have a higher basal adenylate cyclase activity in the presence of GTP and the enzyme is more susceptible to stimulation by hydrolysis-resistant analogues of GTP in the absence of hormones (see figs 2.8 and 4.1 in the current work; Johnson, 1982; Limberd, 1981). During maturation of rat reticulocytes or mouse preadipocytes the amount of the large substrate of cholera toxin decreases relative to the small one. This process parallels changes in the basal activity of adenylate cyclase and in its sensitivity to hydrolysis-resistant analogues of GTP (Larner & Ross, 1981; Lai et al., 1981b). These changes again suggest that the larger substrate of cholera toxin is more potent than the polypeptide of $M_r=42$ 000 in stimulating the exchange of guanyl nucleotides in the absence of hormones (see Limberd, 1981).

In the current work there was no direct evidence showing that cholera toxin inhibits the activity of a GTPase which regulates adenylate cyclase (see section 4.2.5), but work in other systems

suggested that this was almost certainly the mechanism of action of cholera toxin (see section 1.3.3). Adenylate cyclase activity was stimulated to a greater extent by p(NH)ppG alone than by cholera toxin alone (table 4.11). This suggested that cholera toxin did not completely inhibit the GTPase activity of the regulatory component of adenylate cyclase. (p(NH)ppG is not hydrolyzed by the enzyme (Pfeuffer & Helmreich, 1975).) These conclusions would be in agreement with the results of experiments in systems where the GTPase activity can be measured directly (Cassel & Selinger, 1977; Svoboda *et al.*, 1981a; Lester *et al.*, 1982). The high endogenous GTPase activity makes measurement of the adenylate cyclase-associated GTPase impossible in rat liver (see section 4.2.5.1 and Doberska *et al.*, 1980).

In the experiments which are described in section 4.2 the effects of various treatments on the activation of adenylate cyclase by cholera toxin paralleled the effects of ADP-ribosylation. This was consistent with the hypothesis that the stimulation of adenylate cyclase by cholera toxin involved ADP-ribosylation (section 1.3.3).

p(NH)ppG failed to block ADP-ribosylation by cholera toxin, showing that the sites of labelling were not in the binding sites for guanyl nucleotides on the regulatory protein (see section 4.2.7). This result correlated with the observation that cholera toxin modifies the function of initiation factor 2 (eIF-2) in protein synthesis by ADP-ribosylating the β -subunit. The α -subunit of eIF-2 binds GTP (Cooper *et al.*, 1981). These facts suggest a general mechanism for the modification of regulatory GTPases by cholera toxin at sites where guanyl nucleotides are not bound.

The conclusion that cholera toxin ADP-ribosylated the regulatory protein at sites where guanyl nucleotides were not bound was supported by the observation that $[8-^3H]p(NH)ppG$ and $[adenylate-^{32}P]$ ADP-ribose were associated with the same

fractions on gel-permeation chromatography and ion-exchange chromatography (figs 4.10(e) and 4.15). But there was more $[8-^3H]p(NH)ppG$ than $[adenylate-^{32}P]$ ADP-ribose associated with these peaks, therefore both ligands may not be attached to the same protein.

After solubilization from rat liver membranes, the regulatory component of adenylate cyclase eluted from gel-permeation columns with an apparent $M_r \approx 180\ 000$ (section 4.2.6). The value for the M_r is an estimate of the size of a complex containing the regulatory factor and possibly other proteins, detergent and lipid. An estimation of the amount of detergent bound could be made by ultracentrifugation to sediment the protein in sucrose density gradients in H_2O and in deuterated water (Howlett & Gilman, 1980).

The regulatory component of adenylate cyclase has been solubilized from avian erythrocytes and had an apparent $M_r \approx 230\ 000$ (Pfeuffer & Helmreich, 1975). A similar protein was purified free of lipid and detergent from rabbit liver and had an $M_r \approx 132\ 000$ (Northup *et al.*, 1980). The range of apparent M_r -values in the current work was 150 000 to 210 000. The substrate of cholera toxin was solubilized from human erythrocyte membranes using various reagents. The susceptibility of the protein to different procedures for solubilization suggested that it was an elongated protein which was partially buried in the membrane (Kaslow *et al.*, 1980). An elongated protein would have an anomalously high apparent M_r on gel-permeation chromatography and on ultracentrifugation. The differences in M_r -values for the protein are probably also due to the binding of detergent & lipid, and aggregation with other proteins (e.g. the factor which confers sensitivity to cholera toxin, see Pinkett & Anderson (1982) and Schleifer *et al.* (1982)). Therefore the estimate of M_r for the regulatory protein which was obtained in the current work agrees with the values which were reported by other workers who used different sources of the protein.

The regulatory component of adenylate cyclase was a very small proportion of the protein in the rat liver membranes (table 4.17). This observation is in agreement with the results of other workers using rat liver (Doberska *et al.*, 1980) and rabbit liver (Northup *et al.*, 1980). In the current work the agreement between the results using different techniques to measure the amounts of the regulatory protein was quite good. On gel-permeation chromatography the peak which contained the regulatory component of adenylate cyclase was labelled with about one hundred times as much $[8-^3H]p(NH)ppG$ (i.e. 14pmol/mg membrane protein) than $[adenylate-^{32}P] ADP-ribose$ (126fmol were incorporated per mg membrane protein) (see fig. 4.10(e)). The membranes were incubated with sufficient $[8-^3H]p(NH)ppG$ and $[adenylate-^{32}P]NAD^+$ to saturate all of the labelling sites (see figs 4.4 and 4.16). Welton *et al.* (1977) found that rat liver plasma membranes bound one hundred times as much $p(NH)ppG$ than was required for maximal activation of adenylate cyclase. Similar results were obtained by Baker and Potts (1981) using plasma membranes from dog cardiac tissue. It was suggested that there was an excess of the regulatory factor over the catalytic protein from adenylate cyclase in the membranes. This hypothesis would also explain the observation that detergent solubilized only a low proportion of the regulatory protein from rat pancreatic membranes, but most of the adenylate cyclase activity was released under the same conditions (Svoboda *et al.*, 1981b). In the current work similar concentrations of $p(NH)ppG$ were required for maximal activation of adenylate cyclase (fig. 2.8) and for maximal binding to proteins in the membranes (fig. 4.16). This suggested that the affinity of the binding sites which produced activation of adenylate cyclase for $p(NH)ppG$ was similar to the affinity of the hypothetical excess regulatory protein for the analogue.

Pretreatment with $1\mu M NAD^+$ in the presence of cholera toxin was sufficient to maximally stimulate the adenylate cyclase (fig. 4.3). But ADP-ribosylation of the regulatory protein

Table 4.17

The amount of the regulatory component of adenylate cyclase in membranes

The experimental details are given elsewhere. Estimates of the amount of protein are accurate to within $\pm 10\%$. Similar results were obtained when the experiments were repeated.

<u>source of protein</u>	<u>technique for measuring the protein</u>	<u>amount of protein</u>	<u>reference</u>
<u>crude preparation of membranes from rat liver</u>	(a) labelling with cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$, then SDS-PAGE and autoradiography	66fmol/mg membrane protein*	fig. 4.4 current work
	(b) labelling with cholera toxin and γ -adenosine-5',8- ^3H /NAD $^{+}$, then SDS-PAGE, gel-slicing and scintillation-counting	60fmol/mg membrane protein*	fig. 4.2 current work
	(c) labelling with cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$ then gel-permeation chromatography and scintillation-counting	63fmol/mg membrane protein*	fig. 4.10 current work
<u>plasma membranes from rat liver</u>	labelling with cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$, then SDS-PAGE, gel-slicing and scintillation-counting	90pmol/mg plasma membrane protein \pm	Doberska <u>et al.</u> (1980)

Table 4.17 continued on page 166.

Table 4.17 (continued)

<u>plasma membranes</u>	protein purified to	1pmol/mg	Northup <u>et al.</u> ,
<u>from rabbit liver</u>	near homogeneity then	plasma	(1980)
	amount of protein	membrane	
	measured by method of	protein	
	Lowry <u>et al.</u> (1951)		

*This figure assumes that each protein molecule is ADP-ribosylated at two sites (i.e. once on each polypeptide that is a substrate of cholera toxin, see section 5.3.2).

It was taken that ADP-ribosylation occurred at one site on each molecule of protein.

In the current work the amount of regulatory protein is quoted per mg total membrane protein, but other reports state the amount per mg plasma membrane protein.

by cholera toxin increased until the NAD^+ concentration reached $10\mu\text{M}$ (fig. 4.4). Therefore it seemed likely that cholera toxin ADP-ribosylated more regulatory protein than was required to maximally activate the catalytic unit of adenylate cyclase. This provided further evidence suggesting that there was an excess of the regulatory factor over the catalytic protein from adenylate cyclase in the membranes.

Even under optimal conditions, pretreatment with cholera toxin and $\gamma\text{-adenylate-}^{32}\text{P}/\text{NAD}^+$ modified less regulatory protein than was apparently bound by $\gamma\text{-}^3\text{H}/\text{p}(\text{NH})\text{ppG}$. This was probably because the extent of ADP-ribosylation was limited by the amount of non-ADP-ribosylated cofactor protein, rather than by the amount of the substrate which was present (Schleifer *et al.*, 1982; Pinkett & Anderson, 1982).

The two polypeptides which were substrates of cholera toxin fractionated together on ion-exchange chromatography, but the protein which contained these polypeptides eluted at two different concentrations of NaCl (fig. 4.5). This was probably because the cholera toxin-substrates occurred in aggregations which differed in content of protein, lipid or detergent. ADP-ribosylation of the regulatory component of adenylate cyclase in membranes from rat kidneys by cholera toxin required a cofactor protein of $M_r \approx 13\ 000$. This protein occurred in plasma membranes (Pinkett & Anderson, 1982; Schleifer *et al.*, 1982). This factor was easily dissociated from the membranes and from the rest of the adenylate cyclase complex (Pinkett & Anderson, 1982; Schleifer *et al.*, 1982). It could be that two forms of the regulatory protein were produced after solubilization and that one of the forms was associated with the cofactor which confers sensitivity to cholera toxin (see above). As the cofactor is only small ($M_r \approx 13\ 000$), both forms would elute together on gel-permeation chromatography (see figs 4.10 and 4.11) as this technique has

a low resolution. Alternatively one of the two forms seen on ion-exchange chromatography may be an aggregation containing regulatory protein and which is deficient in a molecule which may or may not be a component of adenylate cyclase, but which is not a substrate of cholera toxin. This deficiency could arise from dissociation during the ion-exchange chromatography, explaining why only one form was detected on gel-permeation chromatography (figs 4.10 and 4.11).

Agents which stimulated adenylate cyclase caused an increase in susceptibility to inactivation by phenylglyoxal (table 4.12), suggesting that the enzyme was dissociating or changing conformation to make a larger number of residues available for reaction. If adenylate cyclase was dissociating this would be in agreement with the results of radiation-inactivation experiments (Schlegel et al., 1979; Martin et al., 1979, 1980, see section 1.3.2), and with the observation that treatment with stimulatory guanyl nucleotides increased the susceptibility of the regulatory component of adenylate cyclase to digestion by trypsin (Hudson et al., 1981). Pretreatment with guanyl nucleotides which resist hydrolysis also caused a decrease in the sedimentation coefficient of the complex which contained the regulatory protein (Pfeuffer, 1979; Howlett & Gilman, 1980). All of these results suggest that agents, such as cholera toxin, which inhibit the regulatory GTPase cause the dissociation of the complex in which it occurs.

4.3.6 Conclusions

The conclusions from this section of work are as follows. Cholera toxin ADP-ribosylated two polypeptides which were components of a protein which bound $p(NH)ppG$ and which regulated adenylate cyclase. After solubilization from rat liver membranes the protein had an apparent $M_r \approx 180\ 000$. ADP-ribosylation by cholera toxin caused activation of adenylate cyclase. The sites which cholera toxin modified on the regulatory protein were not where guanyl nucleotides

were bound. Cholera toxin ADP-ribosylated arginine, or arginine-like residues in the protein and probably caused dissociation of adenylate cyclase.

5. INVESTIGATION OF THE STRUCTURES OF THE POLYPEPTIDE SUBSTRATES OF CHOLERA TOXIN

5.1 EXPERIMENTAL

5.1.1 Radiolabelling and separation of the substrates of cholera toxin

Rat liver membranes (about 5mg protein) were incubated in the presence of γ -adenylate- ^{32}P /NAD $^{+}$ with or without cholera toxin (see section 2.6). The membranes were then washed three times and the polypeptides were fractionated by SDS-PAGE (see section 2.8). The final concentration of acrylamide in the separating gel was 8% (w/v). Polypeptides were fixed in the gels as described in table 2.6 except that the fixing solution was changed once so that the total time taken was 30min. Gels were then washed with distilled water and wrapped in a thin sheet of polythene. Each gel was taped to a thin piece of clear plastic which had filter paper attached at several points. A solution was prepared which contained 0.02% (w/v) bromophenol blue and about 200cpm. ^{32}P /μl. This solution was spotted onto the filter paper and then the gel was autoradiographed overnight as described in section 2.9 except that the film was exposed at room temperature. The spots of bromophenol blue were aligned with the black areas on the developed autoradiograph. This enabled excision of the two principal substrates of cholera toxin separately from the gel. Each preparation contained other polypeptides of the same size which were not substrates of cholera toxin. The slices of gel were dried over NaOH for 1h in a vacuum desiccator to remove the acidic fixing solution.

5.1.2 Partial proteolysis of the substrates of cholera toxin and analysis of the products

This experimental technique is based upon that of Cleveland et al., (1977).

An incubation with 250 μ Ci [γ -adenylate- 32 P]NAD $^{+}$ produced enough radiolabelled polypeptides for 4-8 digestions (i.e. 3 000-5 000cpm of each substrate of cholera toxin, 50-150 μ g total protein). Samples from the preparations of the substrates of cholera toxin were analysed by SDS-PAGE (the final concentration of acrylamide in the separating gel was 8% (w/v)) on the same gel as control and toxin-treated membranes, followed by autoradiography (see sections 2.6, 2.8 and 2.9). This showed that the correct polypeptides had been isolated at a sufficient purity (see section 5.2.1, figs 5.2 and 5.4). Slices of gel containing a substrate of cholera toxin (see section 5.1.1) were placed in the sample slots of a second SDS-polyacrylamide gel (see section 2.8). A large stacking gel (about 2cm deep) was used and the final concentration of acrylamide in the separating gel was 15% (w/v). The slices of gel containing the samples were equilibrated with stacking-gel buffer containing 20% (w/v) glycerol, by soaking in it for 30min. The slices were then overlayed with a solution containing a proteolytic enzyme (see section 5.1.3), 0.005% (w/v) bromophenol blue as a marker dye and 10% (w/v) glycerol to increase the density. Samples were electrophoresed until the bromophenol blue reached the top of the separating gel. At this point the substrate of cholera toxin had comigrated with the proteolytic enzyme in the stacking gel. The power was turned off for 30min to allow digestion of the polypeptides and then the products were fractionated by continuing the electrophoresis. In the sample, the substrates of cholera toxin were not pure, but they were the only molecules which were radiolabelled. Therefore the products of partial proteolysis of the substrates of cholera toxin were analysed by autoradiography (see section 2.9). The films were exposed for several weeks (i.e. until the 32 P had decayed to insignificant levels).

5.1.3 Use of proteolytic enzymes

Immediately before addition to the substrates, a solution or suspension of proteolytic enzyme was made at 1mg protein/cm^3 at a pH at which the enzyme was not active. This prevented self-digestion by the enzymes. For most enzymes 1mM HCl was a suitable solvent, but for papain or pepsin, distilled water was used. The specificities of the enzymes are shown in table 5.1.

5.1.4 Exhaustive proteolytic digestion of the substrates of cholera toxin

5.1.4.1 Preparation and handling of equipment

All of the equipment which was used in these investigations was washed with 35% (v/v) HNO_3 for 16h at room temperature. This procedure inactivated any proteolytic enzymes which were present. The equipment was thoroughly rinsed with distilled water before use. During experiments the equipment was handled with gloves to prevent introduction of proteolytic enzymes.

5.1.4.2 Extraction of polypeptides from gels

This experimental technique is based upon that of Tolan et al. (1980).

Slices of gel containing a ^{32}P -labelled substrate of cholera toxin were prepared as described in section 5.1.1. Each slice was treated with 10cm^3 50mM triethanolamine containing 1% (w/v) SDS. This extraction buffer also contained sodium azide, PMSF and benzamidine (see section 2.7), and was adjusted to pH 8.0 with HCl before addition to the gel slices. The slices were incubated with the extraction buffer at room temperature for 1h to allow the gel to swell and then

Table 5.1

Specificities of proteolytic enzymes

The details of experiments in which proteolytic enzymes were used are given in section 5.1. The information in this table is taken from a review by James (1980).

<u>enzyme</u>	<u>source</u>	<u>specificity*</u> (carboxy terminal side of)
V8-protease	<u>Staphylococcus aureus</u>	glutamate (aspartate)
elastase	procine pancreas	alanine, glycine (valine, serine, threonine)
α -chymotrypsin	bovine pancreas	tyrosine, phenylalanine tryptophan
pepsin	porcine stomach mucosa	low specificity, mainly either side of tyrosine, phenylalanine, tryptophan
DCC-trypsin [‡]	bovine pancreas	arginine, lysine
papain	<u>Papaya latex</u>	low specificity, mainly aspartate

*enzymes have low specificities for the residues which are shown in brackets. [‡]The suppliers had treated the trypsin with diphenyl carbamyl chloride to inhibit chymotryptic-like activities.

they were macerated with a glass-rod. The suspension was heated to 65°C for 60min and then shaken at room temperature overnight. Fragments of gel were removed by filtration through glass-wool and then the tubes were each rinsed with an additional 2cm³ extraction buffer. Proteins were precipitated by addition of 5 volumes of acetone containing 0.1M HCl followed by incubation on ice for 30min. The precipitates were collected by centrifugation at room temperature and about 1 000g_{max} for 2min. The proteins were washed with a further two volumes of acidified acetone and then resuspended in two volumes of 10% (w/v) trichloroacetic acid and incubated at 0°C for 30min. The precipitates were pelleted by centrifugation (as above) and then washed twice with ice-cold acetone. The proteins were dried over NaOH in a vacuum desiccator for 30min. Each preparation contained 0.05-0.1mg protein.

5.1.4.3 Carboxyamidomethylation of polypeptides

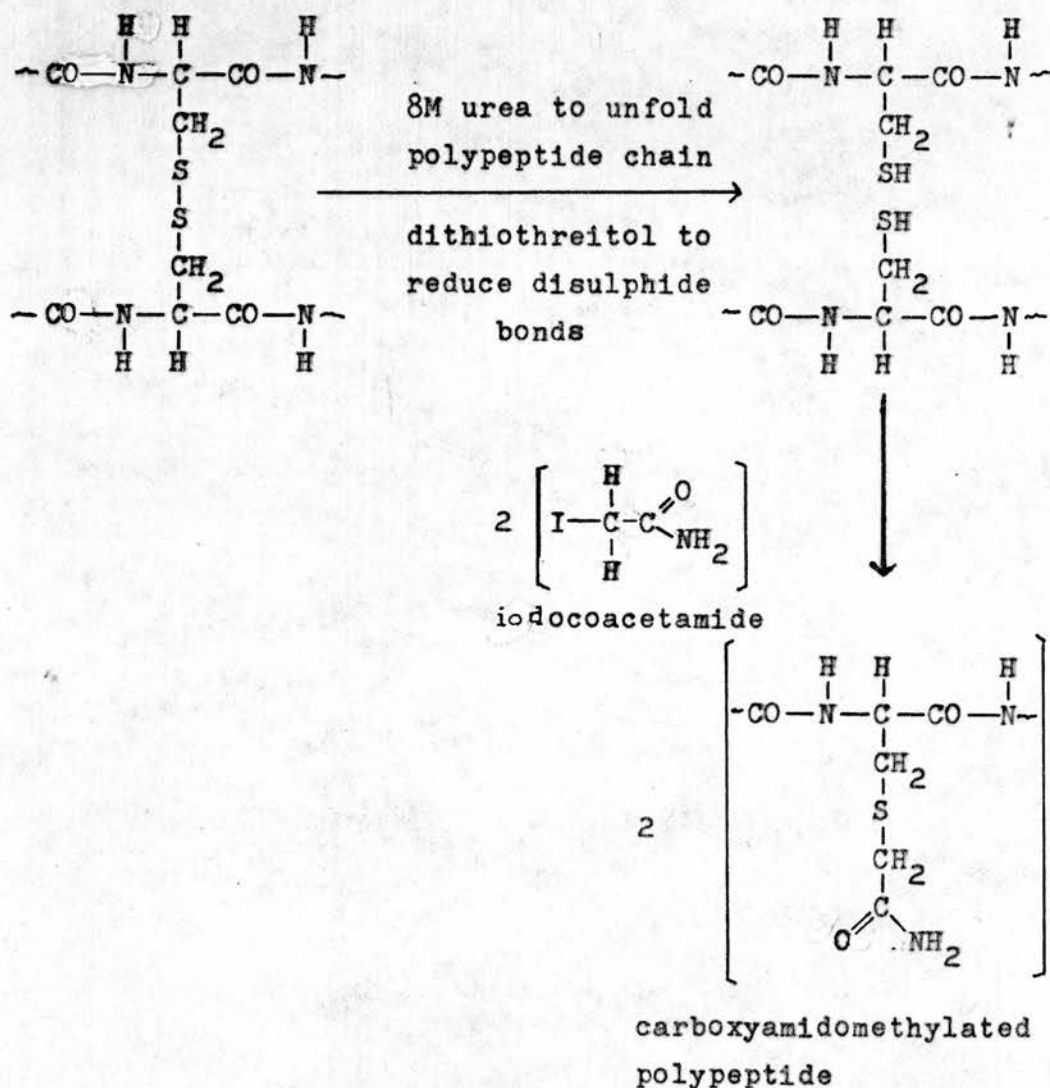
When digested polypeptides are to be analysed by "peptide-mapping" (see section 5.1.5), it is best to have chemically blocked the sulphydryl groups in the sample. This prevents re-association, via disulphide links, of the fragments which are produced by digestion. Such re-association may be incomplete and between different combinations of fragments and could therefore generate artificial heterogeneity in the products. In the current work the polypeptide chains were unfolded in a reducing environment to expose all of the cysteine residues and then the sulphydryl groups were reacted with iodoacetamide (Glazer et al., 1975; Cecil & McPhee, 1959) (see fig. 5.1). The reagents are used in excess and this procedure is assumed to ensure that the carboxyamidomethylation of cysteine residues reaches completion (Glazer et al., 1975; Cecil & McPhee, 1959).

100mM tris containing 8M urea, PMSF, benzamidine and sodium

Fig. 5.1

Carboxyamidomethylation of polypeptides

The experimental procedure for this reaction is given in section 5.1.4.3.



azide (see section 2.7) was adjusted to pH 8.5 with HCl and then degassed and saturated with nitrogen. Polypeptides were resuspended at 0.5-1mg protein cm^{-3} in this buffer. Dithiothreitol was added from a 40mM stock solution in the tris/urea buffer (see above) to a final concentration of 4mM. This was taken to be at least twice the concentration of sulphydryl groups which were associated with proteins in the solution. The preparations were stored for 1h under nitrogen below atmospheric pressure and at room temperature to allow reduction of the proteins to occur (see fig. 5.1). Iodoacetamide was added from a 0.5M stock solution in the tris/urea buffer (see above) to a final concentration of 20mM. Neither the addition of dithiothreitol, nor of iodoacetamide, changed the pH of the mixtures. The incubations were stored at room temperature, under nitrogen and in the dark (as iodoacetamide is sensitive to light,) for 60min with occasional shaking. The reaction was stopped, and excess iodoacetamide was inactivated, by addition of 2-mercaptoethanol to 5-10% (v/v). Then the preparations were dialysed exhaustively against a volatile buffer (see section 5.1.4.4). The first dialysis was in the dark. The carboxyamidomethylated proteins precipitated in the absence of urea.

5.1.4.4 Digestion of polypeptides

An incubation with 250 μ Ci [γ -adenylate- ^{32}P]/NAD $^{+}$ produced enough radiolabelled polypeptides for one digestion (i.e. 500-2 000 c.p.m. of each substrate of cholera toxin). Each preparation of radioactive protein was contaminated with 10-50 μ g of protein that was not labelled. Polypeptides were digested in volatile solvents (see table 5.2) as this allowed the use of freeze-drying to concentrate the products and to remove the solvent before "peptide-mapping". The substrates of cholera toxin produced fragments which were detected by autoradiography, therefore excess protease could be used for the digestion as the enzyme was not

Table 5.2

Volatile media which were used during digestion of polypeptides

Details of the experiments are given in section 5.1.4.4.

<u>media</u>	<u>pH adjusted with</u>	<u>final pH</u>	<u>proteolytic enzymes used</u>
5% (v/v) formic acid	not adjusted	not measured	pepsin
50mM acetate	ammonia	8.5	V8-protease, α -chymotrypsin, DCC-trypsin, elastase
0.2M pyridine	acetic acid	5.5	papain*

*2 μ l 2-mercaptoethanol was added to the preparation before the enzyme. After addition of papain the mixture was flushed with nitrogen and sealed with a stopper.

The volume of the digestion mixture was about 100 μ l.

radioactive. Each enzyme was added to 3% (w/w relative to the substrates) at the start of the digestion and then followed by a further 3% (w/w) 4h later. The complete incubation lasted 16h at 37°C and was stopped by boiling for 1min. The buffer was removed by freeze-drying three times, and the peptides were resuspended in distilled water each time. Before the final freeze-drying the preparations were centrifuged at room temperature and about 10 000g_{max} for 5min to enable removal of insoluble material.

5.1.5 Two-dimensional "peptide-mapping"

Fragments which were produced by proteolytic digestion of polypeptides were analysed by "peptide-mapping" on thin layers of silica-gel (0.25mm x 9cm x 9cm) (Polygram Sil G from Camlab, Cambridge, U.K.) as described by Bates et al. (1975).

The purpose of this separation was to compare the positions that the radiolabelled fragments moved to on different plates. Therefore markers were applied to the plates at the sample positions before the digests were spotted on (see table 5.3). Fragments were electrophoresed in 0.5% (v/v) pyridine, 5% (v/v) acetic acid (this mixture had a pH of 3.5) as the first dimension of the separation. The plates were dried by blowing with cold air and then chromatographed in a mixture of butan-1-ol, acetic acid, water and pyridine (15:3:12:10 by volume) as the second dimension of the "peptide-mapping". Digests which were produced from each substrate of cholera toxin were analysed on separate plates. Two "peptide-maps" were run synchronously: one for each digest. After "peptide-mapping" the plates were dried in a stream of cold air and then autoradiographed for several weeks (see section 2.9).

Table 5.3

Markers used for "peptide-mapping"

The procedure for "peptide-mapping" is given in section 5.1.5. *The markers were gifts from Dr A. P. Ryle, Department of Biochemistry, University of Edinburgh.

<u>marker</u>	<u>concentration of stock solution</u>	<u>volume used</u>
*dnp-aspartate) *dnp-lysine)	1mg of each per cm ³ in 50mM NH ₃	0.3µl
*dns-arginine	1mg/cm ³ in acetone	0.3µl
*dns-histidine	1mg/cm ³ in acetone	0.6µl
pontamine sky blue	1mg/cm ³ in methanol	0.2µl
*methyl green	5mg/cm ³ in 1mM HCl	0.1µl
*xylmol FF	5mg/cm ³ in 1mM HCl	0.1µl

Abbreviations. dnp, 2,4-dinitrophenyl. dns, N,N-dimethylamino naphthalene-5-sulphonyl ("dansyl"). All of these markers were either coloured or visible under ultra-violet illumination.

5.2 RESULTS

In rat liver membranes there are only two predominant substrates of cholera toxin (section 4.2.1). Similar results have been reported in various systems (e.g. mouse S49 lymphoma cells, rat hepatoma cells (Hudson & Johnson, 1980) and human fibroblasts (Watkins et al., 1981)). Therefore ADP-ribosylation by cholera toxin has a relatively high specificity. It has been suggested that cholera toxin modifies only two polypeptides in such cells because the substrates have a structural feature in common which determines the specificity of the reaction (Hudson & Johnson, 1980). This possibility was investigated in the current work.

5.2.1 Purification of the substrates of cholera toxin

The substrates of cholera toxin were separated from each other and each was the predominant radioactive molecule in each preparation (section 5.1 and fig. 5.2). Each substrate was contaminated with polypeptides of the same size, but which were not radioactive.

For the experiments using partial proteolysis of the substrates of cholera toxin (section 5.1.2), the molecules were excised in slices of gel (section 5.1.1 and figs 5.2 and 5.4). In the studies using exhaustive proteolysis of the substrates of cholera toxin (section 5.1.4) the polypeptides were eluted from the slices of gel and then a sample was taken from the material which was carboxyamidomethylated (see section 5.1.4 and fig. 5.2).

5.2.2 The products of partial proteolysis of the substrates of cholera toxin

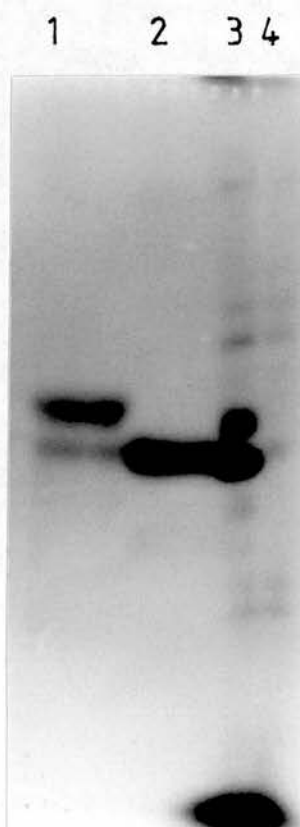
In these experiments (see section 5.1.2) the proteolysis was only partial for several reasons.

Fig. 5.2

Purification of the substrates of cholera toxin from rat liver membranes

Rat liver membranes were incubated with γ -adenylate- ^{32}P /NAD $^{+}$ in the presence or absence of cholera toxin and then the polypeptides were fractionated by SDS-PAGE (see section 5.1.1). The substrates of cholera toxin were purified as described in section 5.2.1 and then analysed by SDS-PAGE, in a gel containing a final concentration of 8% (w/v) acrylamide, followed by autoradiography for several weeks (see sections 2.8 and 2.9).

Lane 1, cholera toxin-substrate of $M_r=47\ 000$. Lane 2, cholera toxin-substrate of $M_r=42\ 000$. Lane 3, membranes treated with cholera toxin. Lane 4, membranes not treated with cholera toxin. The preparations in lanes 1 and 2 were used as samples for exhaustive proteolysis (see fig. 5.6). Similar results were obtained for the purification of substrates of cholera toxin for studies using partial proteolysis (see section 5.1.2 and fig. 5.4).



- (a) Small amounts of proteolytic enzymes were used.
- (b) The proteases were not operating at their pH optima.
- (c) The enzymes were incubated with the substrates for a short time and only at room temperature.
- (d) SDS in the gel decreased the activity of the enzymes.

As the peptides were detected by autoradiography, this technique only detected fragments that contained a site for ADP-ribosylation. Different products may result from cleavages at a number of positions around a single labelling site, or from cleavages around a number of different labelling sites (see fig. 5.3). Therefore, assuming that the substrates had similar structures and few sites of labelling, then the dissimilarities in the products would outweigh the similarities. If there were many sites of ADP-ribosylation on each polypeptide, then the common fragments would predominate.

After digestion with proteolytic enzymes of different specificities (table 5.1), there were some fragments produced which were common to each of the two substrates of cholera toxin, and there were also many fragments which were unique to one or other of the digests (fig. 5.4). These results can be interpreted in two ways.

- (a) There could be many sites of ADP-ribosylation and the substrates are mainly different in structure.
- (b) There may be a few sites of ADP-ribosylation and the substrates have similar structures.

Comparison of the radioactive products of proteolysis of
structurally related polypeptides

This figure is explained in section 5.2.2.

SDS-PAGE and autoradiography to visualise labelled fragments

smaller fragments have a higher mobility on SDS-PAGE

Fig. 5.3 (continued)

(b) Each polypeptide labelled at several sites (*)

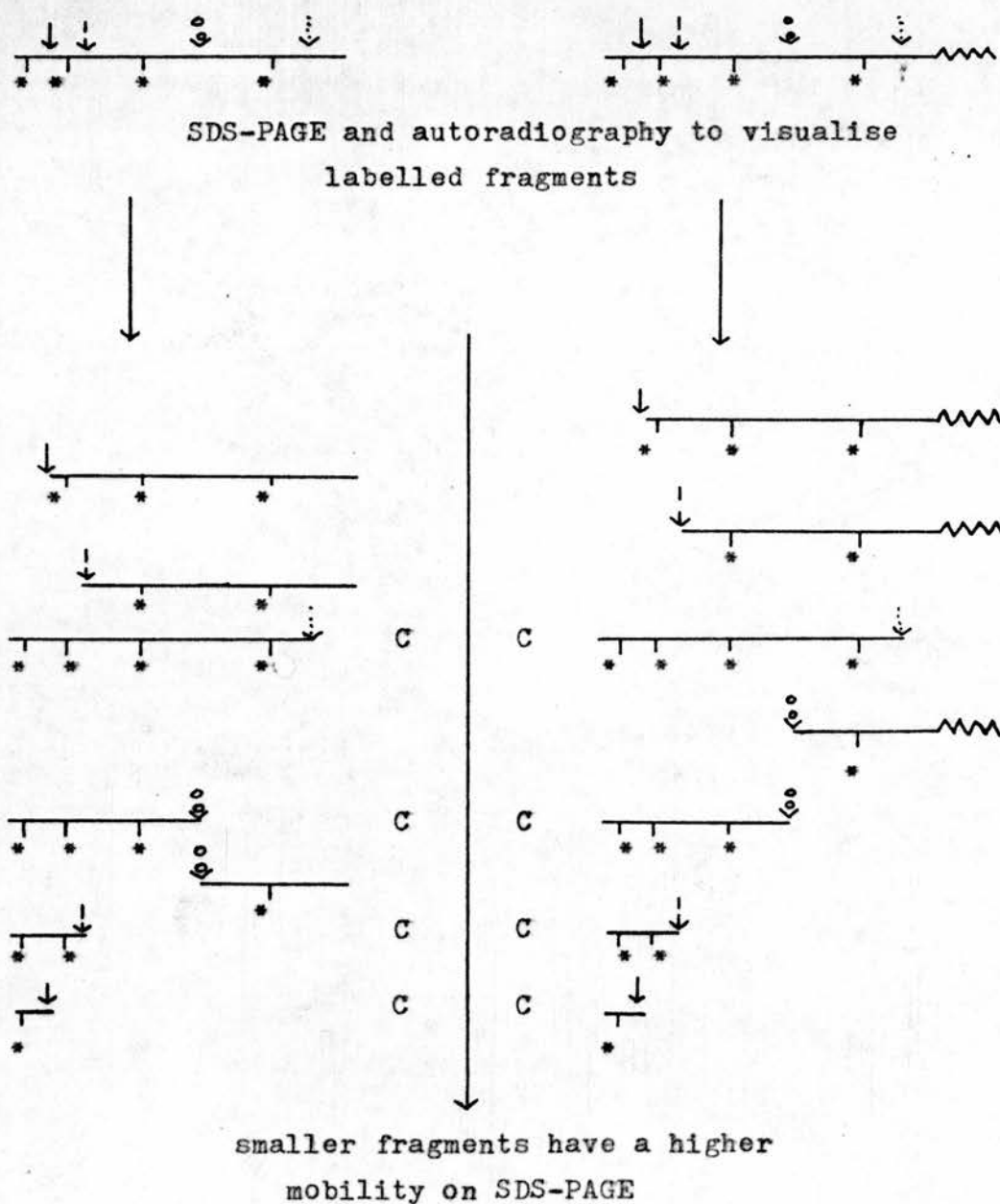


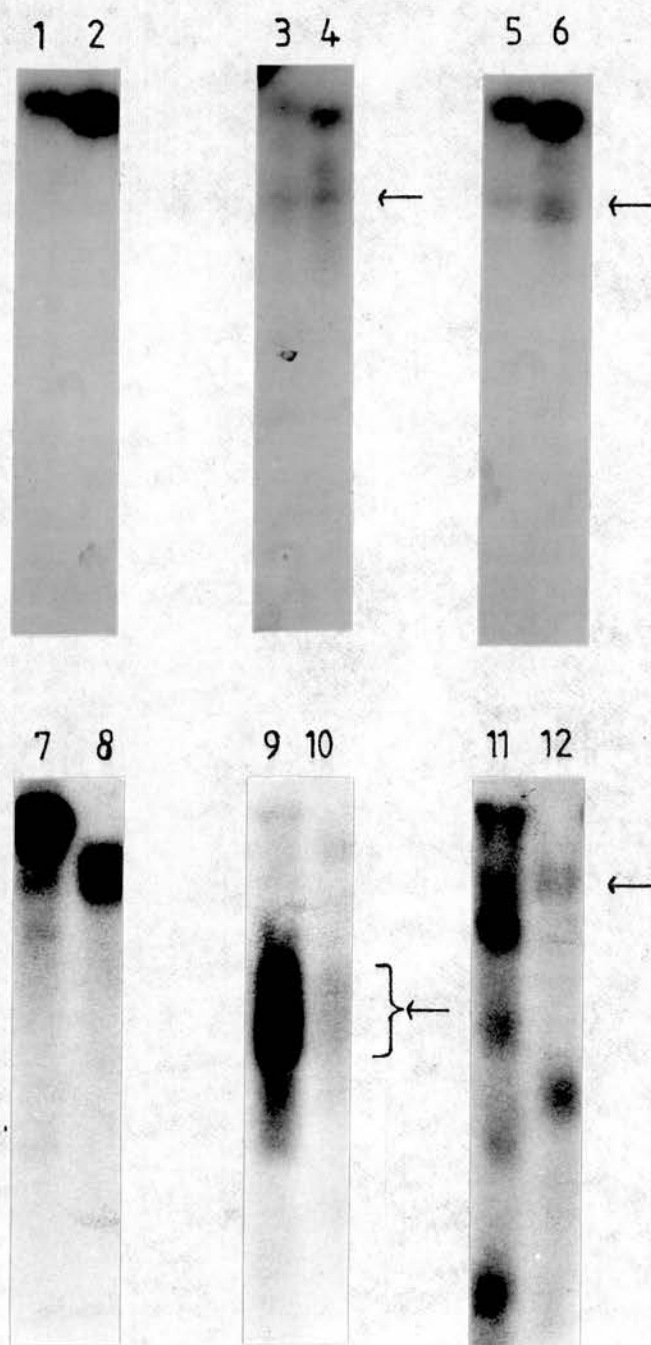
Fig. 5.4

Radioactive products from partial proteolysis of molecules which were
 γ -adenylate- ^{32}P /ADP-ribosylated by cholera toxin

The substrates of cholera toxin were radiolabelled and then purified (see section 5.1.1 and fig. 5.2). The molecules were partially digested and then the products were analysed by SDS-PAGE and autoradiography (see section 5.1.2). Similar results were obtained when the experiments were repeated. Fragments that are common to both substrates are arrowed.

Lanes 1-6, experiment (i). Lane 7-12, experiment (ii). Lanes 1,3,5,7,9 and 11:47 000 M_r -substrate of cholera toxin. Lanes 2,4,6,8,10 and 12:42 000 M_r -substrate of cholera toxin. Lanes 1,2,7 and 8: not digested. Lanes 3 and 4 each digested with 1 μg V8-protease. Lanes 5 and 6 each digested with 1.5 μg elastase. Lanes 9 and 10 each digested with 4 μg V8-protease. Lanes 11 and 12 each digested with 4 μg α -chymotrypsin.

Fig. 5.4



5.2.3 The products of exhaustive proteolysis of the substrates of cholera toxin

To distinguish between the possibilities which are given above, a system was used where proteolysis was exhaustive so that the digestion of the polypeptides approached completion (section 5.1.5). As the time for digestion is increased, cleavage at certain positions reaches completion, but proteolysis at other positions (for which the enzyme has a lower specificity), starts to become significant. Therefore it is difficult to determine an optimum time for incubation to produce material for "peptide-mapping". Each substrate of cholera toxin was digested sequentially by three different enzymes. This was to minimise the probability of producing insoluble "core" polypeptides which would be unsuitable for "peptide-mapping" (Lumsden & Coggins, 1978). This procedure also increased the possibility of digesting each polypeptide between each site of labelling (assuming that the substrates were ADP-ribosylated at more than one site, see fig. 5.5). In "peptide-mapping" the fragments are separated in two dimensions (see section 5.1.5), therefore this procedure has a higher resolution in separating radioactive peptides than the SDS-PAGE technique which was used in section 5.2.2. In "peptide-mapping" the digests from closely related polypeptides would produce identical results unless there was no site for digestion between a site of labelling and the additional sequence on the large polypeptide (fig. 5.5). If this were the case then each digest would contain one unique fragment. If the additional sequence on the large substrate is ADP-ribosylated, then the digest of this molecule would contain radiolabelled peptides which would not be produced from the small substrate. If digestion reached completion between each site of labelling, then this technique could also be used to determine the number of sites of ADP-ribosylation on each substrate (see fig. 5.5).

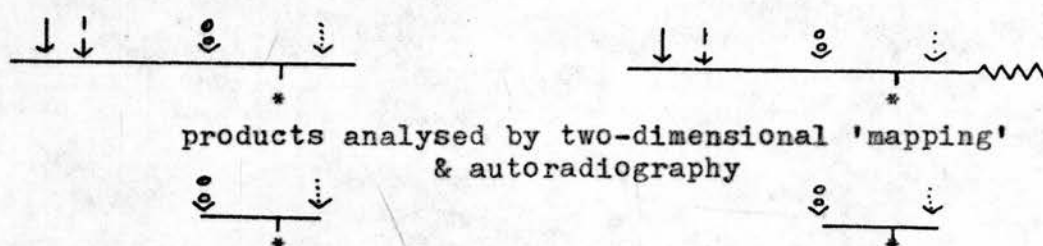
When exhaustive proteolysis followed by "peptide-mapping" was used to investigate the structures of the substrates of cholera

Fig. 5.5

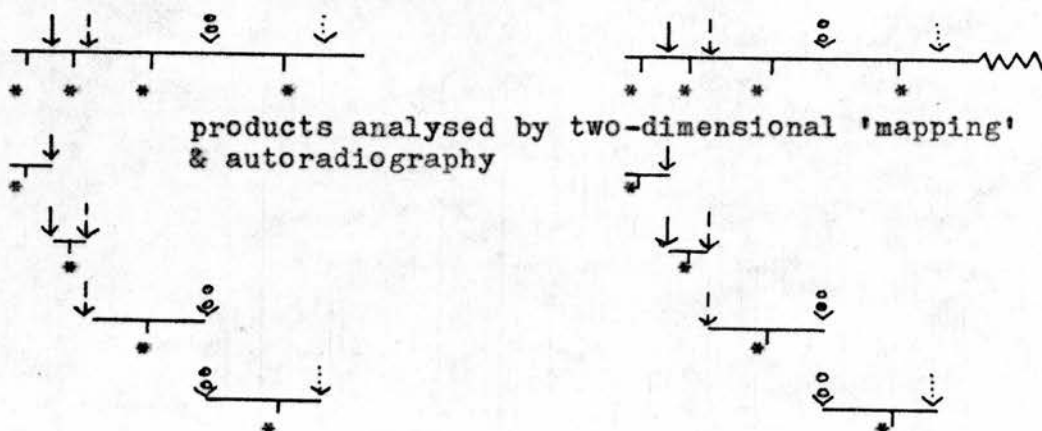
Comparison of the radioactive products of proteolysis of structurally related polypeptides

Digestion is complete (i.e. at ↓, ↓, Ⓢ and Ⓢ). Some of the amino-acid sequence (——) is identical in each substrate, but the larger polypeptide has an extra sequence (~~~~). This figure is explained in section 5.2.3.

(a) Each polypeptide is labelled at a single site (*).



(b) Each polypeptide is labelled at several sites (*).



toxin, almost all of the fragments that were produced were common to both digests (fig. 5.6). This showed that the two substrates of cholera toxin are very similar in structure. There appeared to be one unique fragment (labelled u in fig. 5.6) which occurred in the digest of the smaller substrate of cholera toxin. A radioactive peptide which was uniquely derived from the larger substrate of cholera toxin was not observed. It may be that such a fragment was produced, but that it was not sufficiently labelled to detect, or that it comigrated with another peptide. Alternatively there may be an ADP-ribosylated sequence that only occurs in the polypeptide of $M_r=42\ 000$ (see section 5.3.1).

During "peptide-mapping" some material from each digest remained where the sample was applied. This was insoluble material which had not been removed by centrifugation (see section 5.1.4.4). As this material was not significantly radioactive, it was assumed to not contain any fragments that would have changed the results.

There were two principal radioactive fragments that were produced from each substrate (labelled m in fig. 5.6). Several other radioactive peptides were also detected (fig. 5.6). These molecules probably resulted from incomplete proteolysis, although they may represent sites which were infrequently ADP-ribosylated by cholera toxin.

To test if the ^{32}P -labelled fragments that were derived from each polypeptide comigrated on "peptide-mapping", a digest of a mixture of both substrates of cholera toxin was applied to one "peptide-map" and a digest of the $42\ 000\ M_r$ -substrate of cholera toxin was spotted onto another plate. Both plates were run at the same time. (The material that was digested for each "peptide-map" was analysed by SDS-PAGE, see fig. 5.7.) In this experiment the preparations of the substrates of cholera toxin were digested with proteolytic enzymes which had different specificities to those that were used in the previous investiga-

Fig. 5.6

"Peptide-mapping" of fragments that were produced by exhaustive proteolysis of molecules which were /adenylate-³²P/ADP-ribosylated by cholera toxin

The substrates of cholera toxin were radiolabelled and then purified (see section 5.1.1 and fig. 5.2). The molecules were exhaustively digested sequentially with DCC-trypsin, elastase and finally papain. The products were analysed by "peptide-mapping" and autoradiography (see sections 5.1.4 and 5.1.5). This experiment was only performed once. The arrows indicate the points where the samples were applied. The digest of the polypeptide of $M_r=42\ 000$ was applied to the left hand plate, and the peptides derived from the other substrate of cholera toxin were analysed on the right hand plate. The first dimension of the separation (i.e. electrophoresis) was horizontal and the second dimension (i.e. chromatography) was vertical. The position which the solvent front reached in the chromatography is shown in fig. 5.6(b).

fig. 5.6(a). Autoradiograph of "peptide-maps" of digests of the substrates of cholera toxin.

fig. 5.6(b). Interpretation of the autoradiograph and positions of the markers on "peptide-maps" of digests of the substrates of cholera toxin.

1, dns-histidine. 2, dnp-lysine. 3, dns-arginine. 4, dnp-aspartate. 5, xylmol.FF. 6, pontamine sky blue. 7, methyl green. Further information about the markers is given in table 5.3. m, major radioactive fragment. u, peptide that was unique to one of the digests.

Fig. 5.6(a)

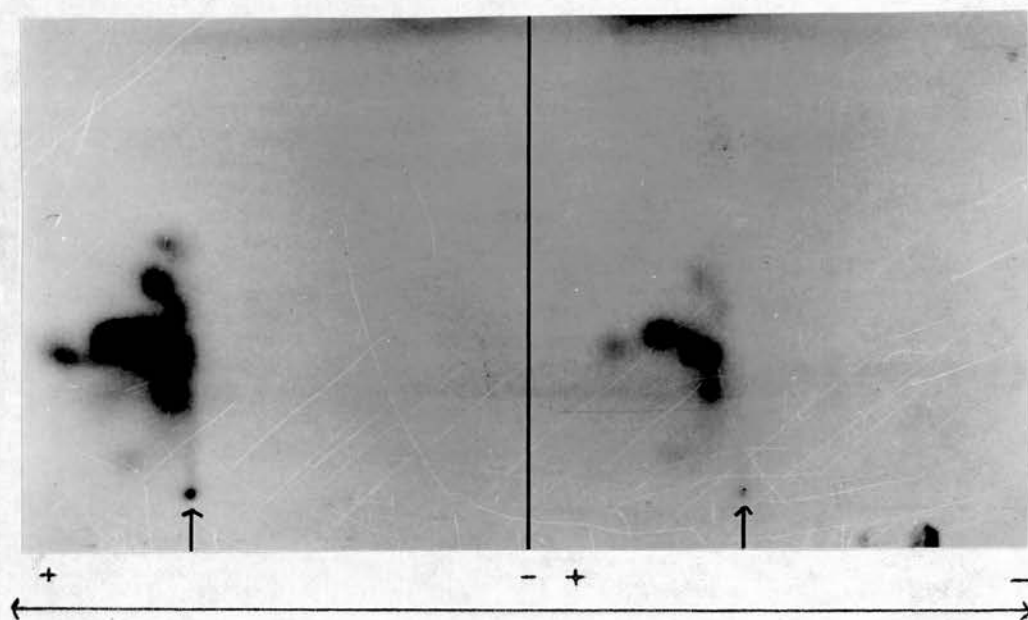


Fig. 5.6(b)

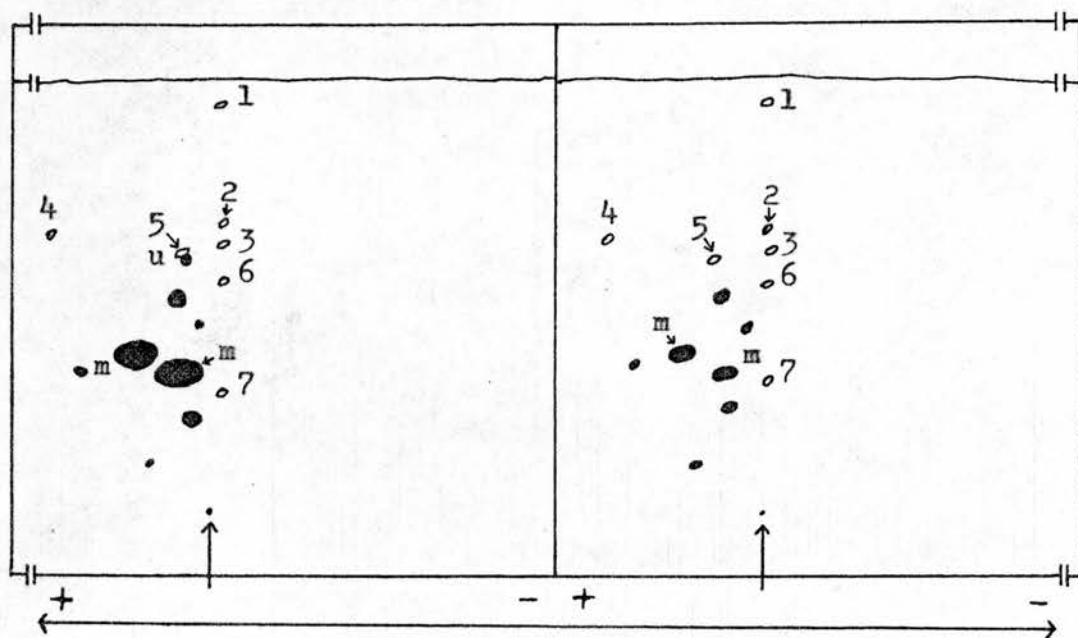
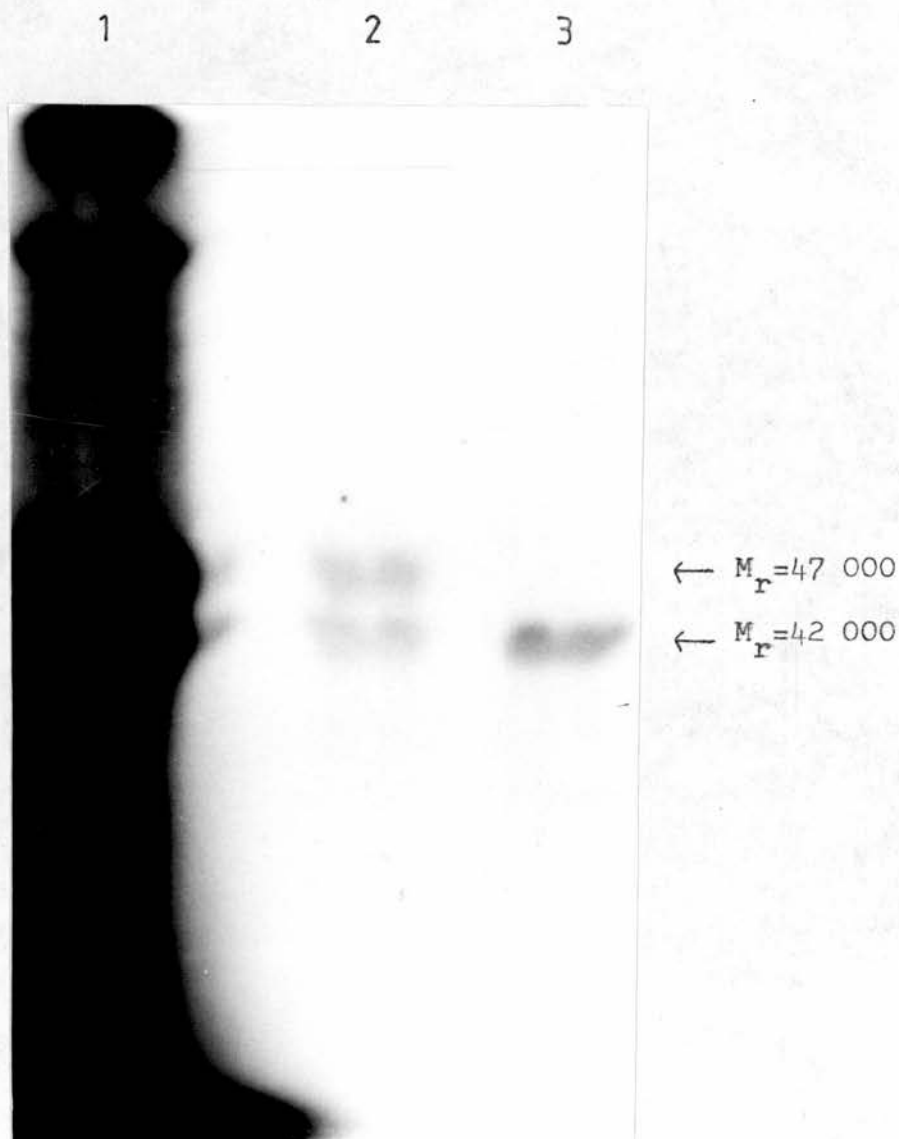


Fig. 5.7

Analysis of the samples that were digested for "peptide-mapping"
(see fig. 5.8).

The substrates of cholera toxin in rat liver membranes were radiolabelled and then purified (see section 5.1.1). The preparations were analysed by SDS-PAGE (the final concentration of acrylamide in the separating gel was 8% (w/v)), followed by autoradiography for six weeks (see sections 2.8 and 2.9). This experiment was only performed once. Lane 1, rat liver membranes which were treated with cholera toxin. Lanes 2 and 3, samples for digestion for "peptide-mapping" (see fig. 5.8).



tion (see table 5.1 and fig. 5.8). "Peptide-mapping" again showed that most fragments were common to both digests, but there was one peptide which was unique to the digest of the preparation containing the larger substrate of cholera toxin (fig. 5.8). A radioactive fragment which only occurred in the digest of the small substrate of cholera toxin would not have been detected in this experiment as peptides that were derived from this molecule were applied to both plates. In this experiment four or more of the peptides from each digest were more radioactive than the others (fig. 5.8).

Each of the "peptide-maps" in figs 5.6 and 5.8 was performed under identical conditions, but there was not a radioactive species which moved to the same position in both experiments. This indicated that all of the radioactivity that was detected was attached to peptides rather than being in the form of free ADP-ribose or smaller groups. (If radiolabel had detached from the peptides, then the same groups would be expected in each experiment, therefore they would have migrated to the same positions on "peptide-maps".) Any radioactivity which could have been liberated in this way would have been highly negatively charged and would probably have migrated off the plate during electrophoresis.

Fewer major ^{32}P -labelled peptides were detected in fig. 5.6 than in fig. 5.8. This may be because radioactive fragments migrated together in the first experiment and moved separately in the second one, or it could be because an individual peptide carried more sites of labelling in the first investigation than in the second one. Alternatively incomplete proteolysis (see earlier in section 5.2.3) may have produced more artefactual heterogeneity in fig. 5.8 than in fig. 5.6.

5.2.4 Conclusions

The conclusions from the experiments using "peptide-mapping" are that the two substrates of cholera toxin are very similar

Fig. 5.8

"Peptide-mapping" of fragments that were produced by exhaustive proteolysis of molecules which were γ -adenylate- ^{32}P /ADP-ribosylated by cholera toxin

The substrates of cholera toxin were radiolabelled and then purified (see section 5.1.1 and fig. 5.7). The molecules were exhaustively digested sequentially with pepsin, V8-protease and then α -chymotrypsin. The products were analysed by "peptide-mapping" and autoradiography (see sections 5.1.4 and 5.1.5). This experiment was only performed once. The arrows indicate the points where the samples were applied. Peptides that were derived from a mixture of the two substrates of cholera toxin were analysed on the left hand plate, and the digest of the polypeptide of $M_r=42\ 000$ was applied to the right hand plate (see fig. 5.7). The first dimension of the separation (i.e. electrophoresis) was horizontal and the second dimension (i.e. chromatography) was vertical. The position which the solvent front reached on chromatography is shown in fig. 5.8(b).

fig. 5.8(a). Autoradiograph of "peptide-maps" of digests of the substrates of cholera toxin.

fig. 5.8(b). Interpretation of the autoradiograph and positions of the markers on "peptide-maps" of digests of the substrates of cholera toxin. 1, dns-histidine. 2, dnp-lysine. 3, dns-arginine. 4, dnp-aspartate. 5, xylmol FF. 6, pontamine sky blue. 7, methyl green. Further information about the markers is given in table 5.3.m, major radioactive fragment. u, peptide that was unique to one of the digests.

Fig. 5.8(a)

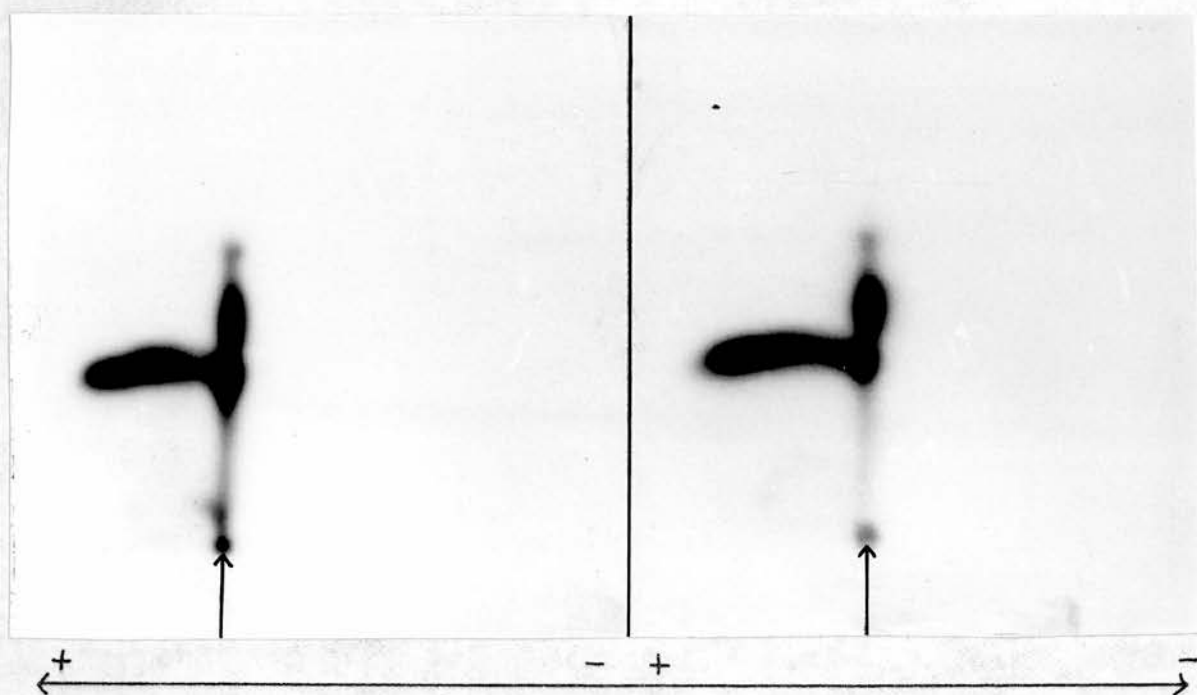
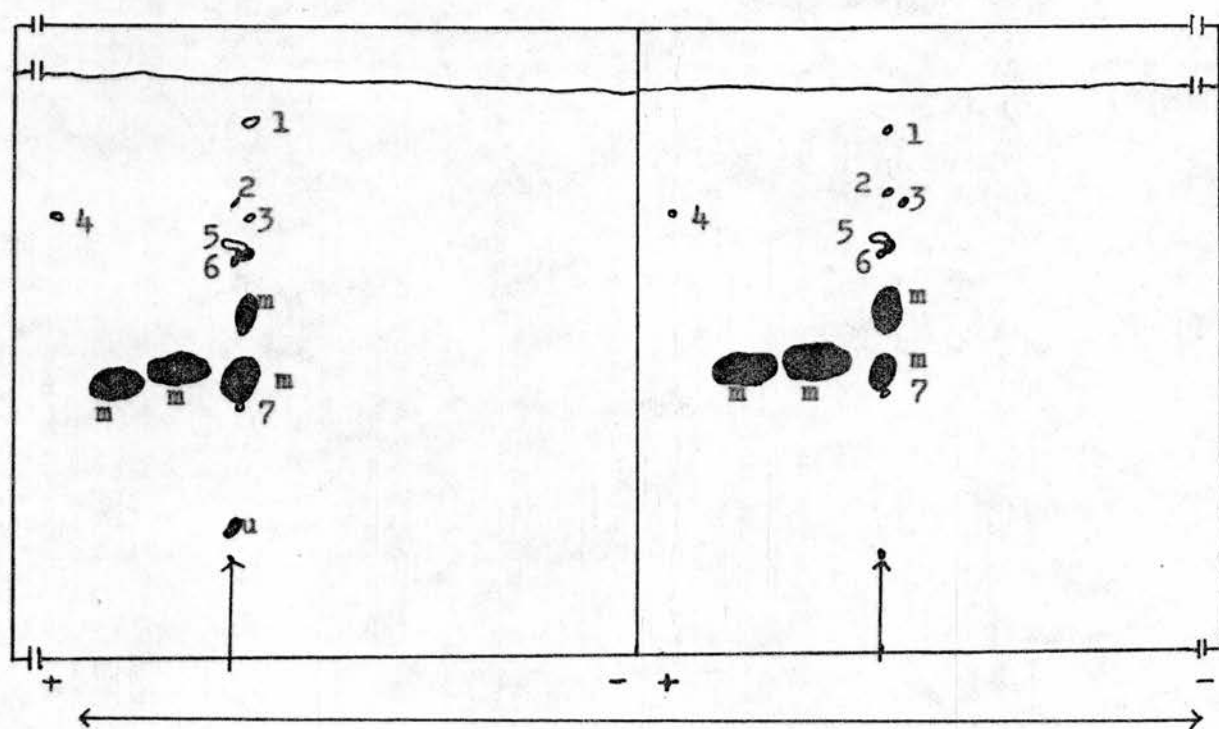


Fig. 5.8(b)



in their structures around the sites of ADP-ribosylation. Each polypeptide is probably modified by cholera toxin at more than one site, but only at a few sites (probably four).

These conclusions are consistent with the results of the experiments using partial proteolysis (section 5.2.2). If some sites were ADP-ribosylated infrequently by cholera toxin, then peptides that contained these sites were probably not detected in the experiments using partial proteolysis. Radiolabelling was detected with less sensitivity in these experiments than in "peptide-mapping" for two reasons.

- (a) Less radiolabelled protein was used in each digest for the partial proteolysis experiments.
- (b) The area that some of the radioactive spots covered was smaller in the experiments using "peptide-mapping".

The digests that were obtained in the experiments using partial proteolysis reflected the structures of most of the substrates, not just the sequences near to the sites of labelling (see section 5.2.2 and fig. 5.3). This was especially the case for the large fragments which had mobilities on SDS-PAGE which were only slightly higher than those of the substrates of cholera toxin (see fig. 5.4). The molecules that were produced from the different substrates of cholera toxin had very similar (or identical) sizes but were only slightly smaller than the undigested material (fig. 5.4). Therefore these experiments showed that the substrates of cholera toxin are very similar in structure throughout their sequences, not just near to the sites of ADP-ribosylation. Exhaustive digestion with three proteolytic enzymes produced a radioactive fragment that was uniquely derived from the substrate of $M_r = 47\ 000$ (fig. 5.8). This observation suggests that at least one of the sites of labelling is on the sequence that only occurs in the larger substrate. Therefore this result also supports the hypothesis that there is more than one site of ADP-ribosylation, at

least on the 47 000 M_r -substrate of cholera toxin.

5.3 DISCUSSION

5.3.1 Similarities between the structures of the substrates of cholera toxin

Hudson and Johnson (1980) used partial proteolysis to study the structures of the substrates of cholera toxin from the membranes of mouse S49 lymphoma cells and HTC4 rat hepatoma cells. Two polypeptides of different sizes were ADP-ribosylated by cholera toxin in each preparation of membranes. In the digests that were produced from these polypeptides, radioactive fragments which were common in size predominated suggesting that the two substrates of cholera toxin had similar structures. In rat fat cells polypeptides of $M_r=42\ 000$, 46 000 and 48 000 were ADP-ribosylated by cholera toxin (Malbon, 1982). The results of experiments using partial proteolysis showed that the two larger substrates of cholera toxin were nearly identical, but the relationship between them and the polypeptide of $M_r=42\ 000$ was unclear (Malbon, 1982).

The results of the current work demonstrated that the two substrates of cholera toxin in rat liver are very similar in structure (see section 5.2.4). This evidence was more conclusive than the published data as, in the current work, proteolysis was exhaustive and the products were separated in two dimensions, rather than only one.

This conclusion suggested that the small substrate of cholera toxin in rat liver may be derived from the larger one by proteolysis. This could explain why only one substrate, of $M_r=42\ 500$, for cholera toxin was detected in plasma membranes from rat liver (Doberska *et al.*, 1980). The large substrate of cholera toxin was probably proteolysed in vitro during purification of the membranes. It is possible that the polypeptide of $M_r=47\ 000$ was the only true substrate of

cholera toxin in rat liver. But two polypeptides in the regulatory component of adenylate cyclase from rabbit liver were ADP-ribosylated by cholera toxin (Northup *et al.*, 1980; Schleifer *et al.*, 1982). Measurements of M_r 's showed that each molecule of regulatory protein contained one copy of each polypeptide (Northup *et al.*, 1980; Sternweis *et al.*, 1981). Therefore it seems likely that two polypeptides (rather than one) were ADP-ribosylated in that protein in rat liver (see section 4.3.5). But the results which are presented in section 5.2 could explain why, if one substrate of cholera toxin was present in excess, it was always the smaller substrate (figs. 4.2 and 4.4; Hudson & Johnson, 1980; Malbon, 1982). Presumably the excess arose from proteolysis of the polypeptide of $M_r=47\ 000$.

The results in section 4.2 show that most, or all, of the amino-acid sequence of the 42 000 M_r -substrate of cholera toxin is in the primary structure of the 47 000 M_r -substrate. The detection of a fragment which could have been unique to the digest of the polypeptide of $M_r=42\ 000$ (fig. 5.6, see section 5.2.3), and the difference in the products of proteolysis of the substrates of cholera toxin with α -chymotrypsin (fig. 5.4) suggest that there may be a short sequence which only occurs in the polypeptide of $M_r=42\ 000$. If this is the case, then the small substrate of cholera toxin could not be produced by proteolysis of the larger one. The polypeptides may have similar structures because the genes which code for them have arisen by divergent evolution from a common ancestral gene as suggested by Hudson and Johnson (1980).

5.3.2 The numbers of sites of ADP-ribosylation by cholera toxin on each polypeptide

The current work showed that cholera toxin probably modified each substrate at more than one site (possibly at four positions) (see section 5.2.3). To measure the number of sites of ADP-ribosylation, digestion must reach completion and must occur

between each of the sites.

Digestion by enzymes must occur under conditions where the protease is active. This means that chaotropic agents (e.g. 6M guanidine, 8M urea) may not be used to completely unfold the substrates, therefore sites for digestion may be hidden leading to incomplete proteolysis. Experiments to determine the numbers of sites of ADP-ribosylation should be performed under fully denaturing conditions. This would restrict the investigation to chemical, rather than enzymic, methods of cleavage of polypeptides. Proteins may be specifically broken down by chemicals at several positions (e.g. methionine residues (Gross, 1967), aspartate-proline linkages (Landon, 1977), cysteine residues (Stark, 1977) and asparagine-glycine linkages (Bornstein & Balian, 1977)). A combination of such techniques could digest at specific positions, and to completion, between all of the sites of ADP-ribosylation on the polypeptides producing fragments which are small enough to be suitable for analysis using "peptide-mapping".

5.3.3 Determination of the specificity of ADP-ribosylation by cholera toxin

The results of the current work showed that the substrates of cholera toxin are very similar in structure (see sections 5.2 and 5.3.1). The recognition of specific features of the molecules by cholera toxin could therefore determine whether a particular molecule is ADP-ribosylated. But the fragments that were radiolabelled by cholera toxin moved to different positions on "peptide-maps" (figs 5.6 and 5.8). This showed that the fragments contained different amino-acids, therefore a long sequence of amino-acids can not dictate which sites on the substrate are ADP-ribosylated by cholera toxin. But it is possible that a short sequence of amino-acids dictates whether cholera toxin modifies a particular site.

Diphtheria toxin has a similar mechanism of action to cholera toxin, ADP-ribosylating elongation factor 2 to inhibit its GTPase activity (see section 1.4). Diphtheria toxin recognises a single amino-acid residue on its substrate, and this residue is ADP-ribosylated by the toxin (Van Ness *et al.*, 1980). The ADP-ribosylated amino-acid is a highly modified histidine residue that is called diphthamide (Van Ness *et al.*, 1980). As this is such an unusual amino-acid, (that it has only been found once, and only in one protein), diphtheria toxin has a very high specificity. But there is no evidence for cholera toxin recognising a modified amino-acid residue. Unlike . . . arginine methyl ester, histidine had no inhibitory effect on the action of cholera toxin (table 4.6). This suggested that cholera toxin may not interact with diphthamide. This is not surprising as cholera toxin is less specific than diphtheria toxin (van Heyningen, 1980).

The regulatory component from adenylate cyclase was purified from rabbit liver. ADP-ribosylation of this protein by cholera toxin was greatly enhanced by addition of another protein which occurred in plasma membranes (Northup *et al.*, 1980; Schleifer *et al.*, 1982). This factor also appears to be required for ADP-ribosylation of the regulatory protein before the substrate is purified from membranes (Pinkett & Anderson, 1982; Kahn & Gilman, 1982). The factor may interact specifically with the regulatory component of adenylate cyclase and with cholera toxin thereby determining the occurrence of ADP-ribosylation without being modified itself.

Cholera toxin only ADP-ribosylates the regulatory protein if the substrate is liganded to GTP, or to an analogue of GTP (see table 4.13 and Schleifer *et al.*, 1982). This suggests that the conformation of the substrate may be important in determining whether ADP-ribosylation occurs.

6. FUTURE WORK

6.1 The action of cholera toxin across membranes

Recent results, including the current work (see sections 1.3.1.2 and 3.3) have suggested that part of cholera toxin interacts with proteins during its transport across membranes. This hypothesis could be tested as follows. Membranes could be treated with cholera toxin and then with a hydrophobic chemical which has the ability to covalently cross-link proteins (e.g. a bifunctional maleimide derivative, aryl halide or isocyanate (see Wold, 1977)). A suitable reagent would dissolve in the membrane and then link the A subunit to any proteins that it interacted with when crossing the membrane.

The role of endocytosis in the uptake of the active part of cholera toxin needs further investigation (see section 1.3.1.2). The time-course of endocytosis of cholera toxin should be compared with that for the activation of adenylate cyclase by the toxin. The mechanism by which cholera toxin could exert its effect across the membrane of the endocytotic vesicle needs to be explained.

6.2 The regulation of adenylate cyclase

Each component of adenylate cyclase should be purified and then the stoichiometry of the holoenzyme may be determined. This is proving to be very difficult (see Ross & Gilman, 1980). The functions of the minor parts of adenylate cyclase (e.g. cytosolic factors) in the regulation of the enzyme are unclear (see section 1.3.2; Ross & Gilman, 1980; Pinkett & Anderson, 1982). Very little is known about the mechanisms by which hormones induce desensitisation of adenylate cyclase. The role of ADP-ribosylation when this process occurs inside cells that have not been treated with cholera toxin is uncertain (see section 1.4). The importance of lipids in the plasma membrane, and of proteins in the cytoskeleton, in the regulation of adenylate cyclase should

be studied (see section 1.3.2.3).

It is not known why the regulatory protein contains two polypeptides which are so similar in structure (see section 5.3.1) and function (see section 4.3.5), especially as only one out of the two polypeptides is required for the activity of the regulatory component (see section 4.3.5). The apparent duplication may be artefactual, arising from proteolysis of the larger polypeptide in vivo and during preparation in vitro (see section 5.3.1). The function and the significance of the two substrates of cholera toxin needs further investigation.

Cholera toxin is proving very useful in studies on the function of the regulatory component of adenylate cyclase. The first step is generally to label the protein using cholera toxin and γ -adenylate- ^{32}P /NAD $^{+}$, but this must modify the activity of the regulatory component. The effects of various treatments on the association of the regulatory protein with other molecules, its structure and conformation can then be measured. The defects in the regulatory protein in S49 UNC cells correlate with the observation that the substrates of cholera toxin in the mutant line were more acidic than the substrates which were derived from the active protein in wild-type S49 cells (Schleifer et al., 1980). In rat reticulocytes, agonists of hormones promoted interaction between the hormone receptor and the regulatory protein which had been labelled using cholera toxin (Limberd et al., 1980). Similar experiments were used to investigate conformational changes in the regulatory protein which were induced by guanyl nucleotides (see section 4.3.5 and Hudson et al., 1981) and functional alterations in the regulatory protein following desensitisation by hormones (Hudson & Johnson, 1981). In studies on the regulation of adenylate cyclase, there are many possibilities for using cholera toxin to specifically radiolabel the regulatory component of the enzyme. Reagents which covalently cross-link proteins (see Wold, 1977) could prove useful to investigate the interactions of the components of the enzyme.

6.3 The activation of adenylate cyclase by cholera toxin

Diphtheria toxin ADP-ribosylates a GTPase to inhibit its activity (see section 1.4). The sequence of the modified peptide, and the structure of the ADP-ribosylated amino-acid are known (Robinson et al., 1974; Van Ness et al., 1978, 1980). Diphtheria toxin and cholera toxin have very similar mechanisms of action, therefore the structures of their substrates may be similar. But the structures of the polypeptides which are ADP-ribosylated by cholera toxin have yet to be completely elucidated.

It is not known how the specificity of ADP-ribosylation by cholera toxin is determined (see section 5.3.3). This may be the role of the protein factor which is required for ADP-ribosylation by cholera toxin, although this factor is not modified itself (Pinkett & Anderson, 1982; Schleifer et al., 1982). The function of this protein needs further investigation. Agents which covalently cross-link proteins (Wold, 1977) could be used to demonstrate interactions of this factor with parts of cholera toxin and parts of adenylate cyclase.

The chemical mechanism of the reaction which is catalyzed by cholera toxin could be studied (see section 1.3.3.1). Investigations into the effect of cholera toxin on adenylate cyclase are not complete (see section 6.2).

6.4 Other experiments using cholera toxin

There are three additional major fields where cholera toxin is useful.

- (a) Parts of cholera toxin have been used as components of hybrid toxins (see section 1.3.1.3).
- (b) cAMP is an important physiological regulator. Cholera toxin has been used extensively to elevate the levels of cAMP in cells and tissues during investigations on the role of cAMP (see van Heyningen, 1977 a).

- (c) The binding specificity of the B subunits of cholera toxin (see section 1.3.1.1) has been used to identify ganglioside GM1 on the surface of cells (see van Heyningen, 1977a).

APPENDIX I - Materials

Buffers, stains, marker-dyes and other reagents were generally of "analar" grade and were purchased from B.D.H. Chemicals Ltd, Poole, Dorset. Lubrol PX, enzymes, substrates and cofactors were usually grade "I" from Sigma London Chemical Co. Ltd, Poole, Dorset. Radiochemicals were purchased from Amersham International plc, Amersham, Bucks.. Scintillation chemicals were bought from Koch-Light Laboratories Ltd, Colnbrook, Bucks., and were of "scintillation" grade. Photographic materials were made by Agfa-Gevaert Ltd, Wimbledon, London and were supplied by H. A. West Ltd, Edinburgh. Chromatography media and paper were purchased from Whatman, Springfield Mill, Maidstone, Kent. For materials which were not of these grades or from these suppliers, the sources are given in the text or below.

V8-protease and DCC-trypsin were bought from Miles Laboratories Ltd, P.O. Box 37, Stoke Court, Stoke Poges, Slough, Berks.. "Dowex" ion-exchange resins were supplied by Sigma (see above).

Cholera toxin was purified by Mrs L. A. Rankine and Dr. S. van Heyningen (using a method which was a modification of that of Mekelanos et al. (1978),) from culture filtrates of Vibrio cholerae kindly grown at the Centre for Applied Microbiology and Research, Porton, Salisbury, Wilts., Mrs L. A. Rankine and Dr. S. van Heyningen isolated the subunits of cholera toxin by gel-permeation chromatography on Sephadex-G75 in urea at low pH (Moss et al., 1976b).

APPENDIX II - Statistical calculation of the correlation between sensitivity to p(NH)ppG or to cholera toxin in different preparations of rat liver membranes

The percent increase in adenylate cyclase activity above basal levels was used for these comparisons to allow for differences in catalytic activities between the preparations (see section 4.2.5.1 and fig. 4.7).

correlation coefficient,	$r = 0.98$
observed variance,	$r^2 = 0.96$
expected variance,	$S(r) = \frac{1-r^2}{n-2} = 0.018$
	$n = \text{number of pairs of observations}$
	$= 4$
variance ratio,	$F = \frac{r^2}{S(r)} = 53.23$
degrees of freedom	$N_1 = 1 \text{ for numerator}$
	$N_2 = n-2 \text{ for denominator}$
	$= 2$

The probability of obtaining this ratio by chance, $P = 0.01-0.05$ (1-5%) (i.e. this is a statistically significant correlation). For an explanation of the statistics see Mather (1964).

APPENDIX III - Abbreviations

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-monophosphate (cyclic AMP)
c.p.m.	counts per minute
CTAB	cetyltrimethylammonium bromide
DCC	treated with diphenyl carbamyl chloride
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DOC	deoxycholate
d.p.m.	decays per minute
DTT	dithiothreitol
E	extinction (wavelength in nm given as subscript)
E.C.	Enzyme Commission number
EDTA	ethylene diaminetetra-acetic acid
EF-2	elongation factor-2
eIF-2	initiation factor-2
GDP	guanosine 5'-diphosphate
GM1	galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminy)- galactosylceramide
ϵ_{\max}	maximum centrifugal force (relative to the gravitational force)
ΔG_t	free energy of transfer from ethanol to water
GTP	guanosine 5'-triphosphate
GTPase	guanosine triphosphatase
GTP γ S	guanosine 5'-(γ -thio)triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
IBMX	isobutylmethylxanthine
MOPS	3-(N-morpholino)propanesulphonic acid
M_r	relative molecular mass
NAD ⁺	nicotinamide adenine dinucleotide
NADase	NAD ⁺ -glycohydrolase
NMN	nicotinamide mononucleotide

nmr	nuclear magnetic resonance
PAGE	polyacrylamide-gel electrophoresis
PMSF	phenylmethylsulphonyl fluoride
p(NH)ppG	β, γ -imido guanosine 5'-triphosphate
R ₃	ratio 3 (see section 3.1.1)
SDS	sodium dodecyl sulphate
tris	2-amino-2-(hydroxymethyl) propane-1,3-diol, (tris)
v/v	volume/volume
w/v	weight/volume
z	discriminant function (see section 3.1.1)

REFERENCES

- Abramowitz, J., Iyengar, R. and Birnbaumer, L. (1979) *Molecular and Cellular Endocrinology* 16, 129-146
- Apps, D. K., Pryde, J. G. and Phillips, J. H. (1980) *Neuroscience* 5, 2279-2287
- Baker, S. P. and Potts, L. T. (1981) *J. Biol. Chem.* 256, 7925-7931
- Barrantes, F. J. (1975) *Biochem. Biophys. Res. Commun.* 62, 407-414
- Bates, D. L., Perham, R. N. and Coggins, J. R. (1975) *Anal. Biochem.* 68, 175-184
- Beckner, S. K. and Blecher, M. (1978) *FEBS Lett.* 95, 319-322
- Beckner, S. K. and Blecher, M. (1981) *Biochim. Biophys. Acta* 673, 477-486
- Beckner, S. K., Blecher, M. and Horner, W. H. (1980) *Fed. Proc. Amer. Soc. Exp. Biol.* 39, 2107
- Bell, R. D. and Doisy, E. A. (1920) *J. Biol. Chem.* 44, 55-67
- Benjamin, R. C. and Gill, D. M. (1980) *J. Biol. Chem.* 255, 10493-10501
- Bennet, V., Mong, L. and Cuatrecasas, P. (1975) *Journal of Membrane Biology* 24, 107-129
- Bergmeyer, H. U. and Brent, E. (1974) in "Methods of enzymatic analysis" (Bergmeyer, H. U. ed.) vol. 2, pp. 574-579, Academic Press, New York
- Bigelow, C. C. (1967) *J. Theor. Biol.* 16, 187-211
- Bock, K. W., Siekevitz, P. and Palade, G. E. (1971) *J. Biol. Chem.* 246, 188-195
- Boquet, P. (1979) *Europ. J. Biochem.* 100, 483-489
- Bornstein, P. and Balian, G. (1977) *Methods Enzymol.* 47, 132-145
- Bradford, M. M. (1976) *Anal. Biochem.* 72, 248-254
- Burton, J. (1956) *Biochem. J.* 62, 315-323
- Cassel, D. and Pfeuffer, T. (1978) *Proc. Natl. Acad. Sci. USA* 75, 2669-2673
- Cassel, D. and Selinger, Z. (1977) *Proc. Natl. Acad. Sci. USA* 74, 3307-3311
- Cassel, D. and Selinger, Z. (1978) *Proc. Natl. Acad. Sci. USA* 75, 4155-4159

Cassel, D., Lefkovitz, H. and Selinger, Z. (1977) Journal of Cyclic Nucleotide Research 3, 393-406

Cassel, D., Eckstein, F., Lowe, M. and Selinger, Z. (1979) J. Biol. Chem. 254, 9835-9838

Cassuto, J., Fahrenkrug, J., Jodal, M., Tuttle, R. and Lundgren, O. (1981) Gut 22, 958-963

Cech, C. Y., Broadus, W. L. and Maguire, M. E. (1980) Molecular and Cellular Biochemistry 33, 69-72

Cecil, R. and McPhee, J. R. (1959) Advan. Protein Chem. 14, 255-389

Chen, L. C., Rohde, J. E. and Sharp, G. W. G. (1972) J. Clin. Invest. 51, 731-740

Cho-Chung, Y. S., Clair, T., Bodwin, J. S. and Hill, J. M. (1980) Biochem. Biophys. Res. Commun. 95, 1306-1313

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102-1106

Cohen, P., Duewer, J. and Fischer, E. H. (1971) Biochemistry 10, 2683-2691

Cooper, D. M. F., Jagus, R., Somers, R. L. and Rodbell, M. (1981) Biochem. Biophys. Res. Commun. 101, 1179-1185

Crawford, A., MacNeil, S., Amirsooli, H., Brown, B. L. and Tomlinson, S. (1981) Biochemical Society Transactions 9, 207P

Critchley, D. R., Ansell, S. and Dilks, S. (1979) Biochemical Society Transactions 7, 314-319

Critchley, D. S., Streuli, C. H., Kellie, S., Ansell, S. and Patel, B. (1982) Biochem. J. 204, 209-219

Dallas, W. S. and Falkow, S. (1980) Nature (London) 288, 499-501

Darnall, D. W. and Klotz, I. M. (1975) Arch. Biochem. Biophys. 166, 651-682

Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427

*

De, S. N. (1959) Nature (London) 183, 1533-1534

De Wolf, M. J. S., Vitti, P., Ambesi-Impiombato, F. S. and Kohn, L. D. (1981) J. Biol. Chem. 256, 12287-12296

Doberska, C. A., MacPherson, A. J. S. and Martin, B. R. (1980) Biochem. J. 186, 749-754

Douglas, M. and Butow, R. A. (1976) Proc. Natl. Acad. Sci. USA 73, 1083-1086

* Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1979) in "Data for Biochemical Research" Oxford University Press

Duffy, L. K., Peterson, J. W. and Kurosky, A. (1981a) FEBS Lett. 126, 187-190

Duffy, L. K., Peterson, J. W. and Kurosky, A. (1981b) J. Biol. Chem. 256, 12252-12256

Evans, E. A. (1976) "Self-decomposition of radiochemicals: principles, control, observations and effects". Radiochemical Centre review no. 16. Radiochemical Centre Ltd, Amersham, Bucks.

Evans, W. H. (1978) in "Preparation and characterization of mammalian plasma membranes". pp. 114-128, North Holland Publishing Co., Amsterdam, New York and Oxford

Evans, D. J., Chen, L. C., Curlin, G. T. and Evans, D. G. (1972) Nature (London) 236, 137-138

Fahrney, D. E. and Gold, A. M. (1963) J. Amer. Chem. Soc. 85, 997-1000

Fernandes, P. B., Welsh, K. M. and Bayer, M. E. (1979) J. Biol. Chem. 254, 9254-9261

Field, M. (1979) Amer. J. Clin. Nutr. 32, 189-196

Fischer, L. (1980) in "Gel-filtration chromatography" 2nd edition pp.159-164, Elsevier-North Holland, Amsterdam, New York and Oxford

Fishman, P. H. (1980a) in "Secretory diarrhoea" (Field, M. et al. eds), pp.85-106, Waverly Press, Baltimore

Fishman, P. H. (1980b) Journal of Membrane Biology 54, 61-72

Fishman, P. H. and Atikkan, E. E. (1980) Journal of Membrane Biology 54, 51-60

Fishman, P. H., Moss, J. and Osborne, J. C. (1978) Biochemistry 17, 711-716

Fishman, P. H., Pacuszuka, T., Hom, B. and Moss, J. (1980) J. Biol. Chem. 255, 7657-7664

Flores, J. and Sharp, G. W. G. (1975) J. Clin. Invest. 56, 1345-1349

Flores, J., Witkum, P. and Sharp, G. W. G. (1976) J. Clin. Invest. 57, 450-458

Franks, D. J., Tunnicliff, G. and Ngo, T. T. (1980) Biochim. Biophys. Acta 611, 358-362

Gill, D. M. (1975) Proc. Natl. Acad. Sci. USA 72, 2064-2068

Gill, D. M. (1976a) Biochemistry 15, 1242-1248

Gill, D. M. (1976b) J. Infec. Dis. 133, S55-S63

- Gill, D. M. (1979) *Journal of Supramolecular Structure* 10, 151-163
- Gill, D. M. and Meren, R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 3050-3054
- Gill, D. M. and Rappaport, R. S. (1979) *J. Infec. Dis.* 139, 674-680
- Gill, D. M., Clements, J. D., Robertson, D. C. and Finkelstein, R. A. (1981) *Infec. Immun.* 33, 677-682
- Glazer, A. N., Delange, R. J. and Sigman, D. S. (1975) in "Chemical Modification of Proteins", pp.103-104, North Holland Publishing Co., Amsterdam and Oxford
- Gross, E. (1967) *Methods Enzymol.* 11, 238-255
- Hagmann, J. and Fishman, P. H. (1981) *Biochem. Biophys. Res. Commun.* 98, 677-684
- Hagmann, J. and Fishman, P. H. (1982) *Biochim. Biophys. Acta* 720, 181-187
- Hanski, E., Sternweis, P. C., Northup, J. K., Dromerick, A. W. and Gilman, A. G. (1981) *J. Biol. Chem.* 256, 12911-12919
- Helenius, A. and Simons, K. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 529-532
- Hilz, H. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* 362, 1415-1425
- Holmgren, J. (1980) in "The Molecular Basis of Microbial Pathogenicity" (Smith, H. et al. eds.), pp.269-284, Weinheim: Verlag Chemie
- Honjo, T., Nishizuka, Y. and Hayaishi, O. (1969) *Cold Spring Harbor Symp. Quant. Biol.* 34, 603-608
- Houslay, M. D. and Elliott, K. R. F. (1981) *FEBS Lett.* 128, 289-292
- Howlett, A. C. and Gilman, A. G. (1980) *J. Biol. Chem.* 255, 2861-2866
- Hudson, T. H. and Johnson, G. L. (1980) *J. Biol. Chem.* 255, 7480-7486
- Hudson, T. H. and Johnson, G. L. (1981) *Mol. Pharmacol.* 20, 694-703
- Hudson, T. H., Roeber, J. F. and Johnson, G. L. (1981) *J. Biol. Chem.* 256, 1459-1465
- Inglewski, B. H., Liu, P. V. and Kabat, D. (1977) *Infec. Immun.* 15, 138-144
- James, G. T. (1980) *Methods Biochem. Anal.* 26, 165-200
- Jeffcoate, S. L. and White, N. (1974) *J. of Clin. Endocrinol. Metab.* 38, 155-157
- Johnson, G. L. (1982) in "Molecular Actions of Toxins and Viruses" (Cohen, P. and van Heyningen, S. eds.), pp.33-49, Elsevier Biomedical Press, Amsterdam and New York

- Johnson, G. L., Kaslow, H. R. and Bourne, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 3113-3117
- Johnson, G. L., Kaslow, H. R., Farfel, Z. and Bourne, H. R. (1980) *Advances in Cyclic Nucleotide Research* 13, 1-37
- Kahn, R. A. and Gilman, A. G. (1982) *Fed. Proc. Amer. Soc. Exp. Biol.* 41, 1139
- Kaslow, H. R., Johnson, G. L., Brothers, V. M. and Bourne, H. R. (1980) *J. Biol. Chem.* 255, 3736-3741
- Kaslow, H. R., Groppi, V. E., Abood, M. E. and Bourne, H. R. (1981a) *J. Cell Biol.* 91, 410-413
- Kaslow, H. R., Cox, D., Groppi, V. E. and Bourne, H. R. (1981b) *Mol. Pharmacol.* 19, 406-410
- Katz, M. S., Kelly, T. M., Pineyro, M. A. and Gregerman, R. I. (1978) *Journal of Cyclic Nucleotide Research* 5, 389-407
- Katz, M. S., Kelly, T. M., Pineyro, M. A. and Gregerman, R. I. (1980) *Biochim. Biophys. Acta* 632, 11-25
- Kempner, E. S. and Schlegel, W. (1979) *Anal. Biochem.* 92, 2-6
- Kimura, N., Nakone, K. and Nagata, N. (1976) *Biochem. Biophys. Res. Commun.* 70, 1250-1256
- King, C. A., van Heyningen, W. E. and Gascoyne, N. (1976) *J. Infec. Dis.* 133, S75-S81
- Klotz, I. M., Darnall, D. W. and Langerman, N. R. (1975) in "The Proteins" (Neurath, H. *et al.*, eds.), vol. I, pp.293-411, Academic Press, London
- Kun, E., Zimmer, P. H., Chang, A. C. Y., Puschendorf, B. and Grunicke, H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 1436-1440
- Kurosky, A., Markel, D. E., Touchstone, B. and Peterson, J. W. (1976) *J. Infec. Dis.* 133, S14-S22
- Laemmli, U. K. (1970) *Nature (London)* 227, 680-685
- Lai, C. Y. (1980) *CRC Crit. Rev. Biochem.* 9, 171-205
- Lai, C. Y., Cancedda, F. and Chang, D. (1979) *FEBS Lett.* 100, 85-89
- Lai, C. Y., Cancedda, F. and Duffy, L. K. (1981a) *Biochem. Biophys. Res. Commun.* 102, 1021-1027
- Lai, E., Rosen, O. M. and Rubin, C. S. (1981b) *J. Biol. Chem.* 256, 12866-12874
- Landon, M. (1977) *Methods Enzymol.* 47, 145-149

- Larner, A. C. and Ross, E. M. (1981) J. Biol. Chem. 256, 9551-9557
- Laskey, R. A. and Mills, A. D. (1977) FEBS Lett. 82, 314-316
- Lee, Y. and Horecker, B. C. (1974) Arch. Biochem. Biophys. 162, 401-411
- Lester, H. A., Steer, M. L. and Levitzki, A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 719-723
- Levinson, S. J. and Blume, A. J. (1977) J. Biol. Chem. 252, 3766-3774
- Levitzki, A. (1981) CRC Crit. Rev. Biochem. 10, 81-112
- Limberd, L. E. (1981) Biochem. J. 195, 1-13
- Limberd, L. E., Gill, D. M., Stadel, J. M., Hickey, A. R. and Lefkowitz, R. J. (1980) J. Biol. Chem. 255, 1854-1861
- Lin, M. C. and Taniuchi, M. (1980) Journal of Cyclic Nucleotide Research 6, 359-367
- Lin, M. C., Welton, A. F. and Berman, M. F. (1978) Journal of Cyclic Nucleotide Research 4, 159-168
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
- Lumsden, J. and Coggins, J. R. (1978) Biochem. J. 169, 441-444
- Malbon, C. C. (1982) Biochim. Biophys. Acta 714, 429-434
- Mannhalter, J. W., Gilliland, D. G. and Collier, R. J. (1980) Biochim. Biophys. Acta 626, 443-450
- Martin, B. R., Houslay, M. D. and Kennedy, E. L. (1977) Biochem. J. 161, 639-642
- Martin, B. R., Stein, J. M., Kennedy, E. L., Doberska, C. A. and Metcalfe, J. C. (1979) Biochem. J. 184, 253-260
- Martin, B. R., Stein, J. M., Kennedy, E. L. and Doberska, C. A. (1980) Biochem. J. 188, 137-140
- Mason, T. L., Poyton, R. O., Wharton, D. C. and Schatz, G. (1973) J. Biol. Chem. 248, 1346-1354
- Mather, K. (1964) "Statistical Analysis in Biology", Meuthen and Co. Ltd, London
- Matuo, Y., Wheeler, M. A. and Bitensky, M. A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2654-2658
- Mekelanos, J. J., Collier, R. J. and Romig, W. R. (1978) Infec. Immun. 20, 552-558

- Mekelanos, J. J., Collier, R. J. and Romig, W. R. (1979) J. Biol. Chem. 254, 5849-5854
- Mendez, E., Lai, C. Y., Wodnar-Filipowicz, A. (1975) Biochem. Biophys. Res. Commun. 67, 1435-1443
- Montesano, R., Roth, J., Robert, A. and Orci, L. (1982) Nature (London) 296, 651-653
- Morita, A., Tsao, D. and Kim, Y. S. (1980) J. Biol. Chem. 255, 2549-2553
- Morrisett, J. D., Jackson, R. L. and Gotto, A. M. (1975) Annu. Rev. Biochem. 44, 183-257
- Moss, J. and Stanley, S. J. (1981) J. Biol. Chem. 256, 7830-7833
- Moss, J. and Vaughan, M. (1977) J. Biol. Chem. 252, 2455-2457
- Moss, J. and Vaughan, M. (1981) Molecular and Cellular Biochemistry 37, 75-90
- Moss, J., Fishman, P. H., Richards, R. A., Alving, C. R., Vaughan, M. and Brady, R. O. (1976a) Proc. Natl. Acad. Sci. U.S.A. 73, 3480-3483
- Moss, J., Manganiello, V. C. and Vaughan, M. (1976b) Proc. Natl. Acad. Sci. U.S.A. 73, 4424-4427
- Moss, J., Osborne, J. C., Fishman, P. H., Brewer, H. B., Vaughan, M. and Brady, R. O. (1977a) Proc. Natl. Acad. Sci. U.S.A. 74, 74-78
- Moss, J., Richards, R. L., Alving, C. R. and Fishman, P. H. (1977b) J. Biol. Chem. 252, 797-798
- Moss, J., Stanley, S. J. and Lin, M. C. (1979a) J. Biol. Chem. 254, 11993-11996
- Moss, J., Garrison, S., Fishman, P. H. and Richardson, S. H. (1979b) J. Clin. Invest. 64, 381-384
- Moss, J., Stanley, S. J., Watkins, P. A. and Vaughan, M. (1980a) J. Biol. Chem. 255, 7835-7837
- Moss, J., Stanley, S. J. and Watkins, P. A. (1980b) J. Biol. Chem. 255, 5838-5840
- Moss, J., Stanley, S. J., Morin, J. E. and Dixon, J. E. (1980c) J. Biol. Chem. 255, 11085-11087
- Mullin, B. R., Alog, S. M., Fishman, P. H., Lee, G., Kohn, L. D. and Brady, R. O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1679-1683
- Mullin, B. R., Fratianne, G. G., and Montanaro, A. J. (1980) Fed. Proc. Amer. Soc. Exp. Biol. 39, 1645
- Nakashima, Y., Napiorkowski, P., Schafer, D. E. and Konigsberg, W. H. (1976) FEBS Lett. 68, 275-278

- Nakazawa, K., Ueda, K., Honjo, T., Yoshihara, K., Nishizuka, Y. and Hayashi, O. (1968) *Biochem. Biophys. Res. Commun.* 32, 143-149
- Newby, A. C. and Chrambach, A. (1979) *Biochem. J.* 177, 623-630
- Newby, A. C., Rodbell, M. and Chrambach, A. (1978) *Arch. Biochem. Biophys.* 190, 109-117
- Nimmo, I. A. and Atkins, G. L. (1979) *Anal. Biochem.* 94, 270-273
- Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M. and Gilman, A. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 6516-6520
- Nozaki, Y. and Tanford, C. (1971) *J. Biol. Chem.* 246, 2211-2217
- Oeltmann, T. N. and Forbes, J. T. (1981) *Arch. Biochem. Biophys.* 209, 362-370
- Ohtomo, N., Muraoka, T., Tashiro, A., Zinnaka, Y. and Amako, K. (1976) *J. Infec. Dis* 133, S31-S40
- Olsnes, S. (1981) *Nature (London)* 290, 84
- Oppenheimer, N. J. (1978) *J. Biol. Chem.* 253, 4907-4910
- Pappenheimer, A. M. and Gill, D. M. (1977) *Science* 182, 353-358
- Peng, C. T. (1977) "Sample preparation in liquid scintillation counting". Radiochemical Centre review no. 17. Radiochemical Centre Ltd, Amersham, Bucks.
- Peytremann, A., Nicholson, W. E., Liddle, G. W., Hardman, J. G. and Sutherland, E. W. (1973) *Endocrinology* 92, 525-530
- Pfeuffer, T. (1977) *J. Biol. Chem.* 252, 7224-7234
- Pfeuffer, T. (1979) *FEBS Lett.* 101, 85-89
- Pfeuffer, T. and Helmreich, E. J. M. (1975) *J. Biol. Chem.* 250, 867-876
- Pilkis, S. J., Exton, J. H., Johnson, R. A. and Park, C. A. (1974) *Biochim. Biophys. Acta.* 343, 250-267
- Pinkett, M. O. and Anderson, W. B. (1982) *Biochim. Biophys. Acta.* 714, 337-343
- Raftery, M. A., Vandleen, R. L., Reed, K. L. and Lee, T. (1975) *Cold Spring Harbor Symp. Quant. Biol.* 40, 193-202
- Reed, R. G., Feldhoff, R. C., Clute, O. L. and Peters, T. (1975) *Biochemistry* 14, 4578-4583
- Reilly, T. M., Beckner, S., McHugh, E. M. and Blecher, M. (1981) *Biochem. Biophys. Res. Commun.* 98, 1115-1120

- Reynolds, J. A., Herbert, S., Polet, H. and Steinhardt, J. (1967) *Biochemistry* 6, 937-947
- Robinson, E. A., Henricksen, Q. and Maxwell, E. S. (1974) *J. Biol. Chem.* 249, 5088-5093
- Rodbell, M. (1980) *Nature (London)* 284, 17-22
- Rodbell, M., Birnbaumer, L., Pohl, S. L. and Krans, H. M. J. (1971) *J. Biol. Chem.* 246, 1877-1882
- Rodbell, M., Lin, M. C., Salamon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M. and Berman, M. (1975) *Advances in Cyclic Nucleotide Research* 5, 3-29
- Rogers, A. W. (1979) "Practical autoradiography". Radiochemical Centre review no. 20, Radiochemical Centre Ltd, Amersham, Bucks.
- Ross, E. M. and Gilman, A. G. (1980) *Annu. Rev. Biochem.* 49, 533-564
- Sahyoun, N. E., Shatila, N., Levine, H. and Cuatrecasas, P. (1981a) *Biochem. Biophys. Res. Commun.* 102, 1216-1222
- Sahyoun, N. E., Levine, H., Davis, J., Hebdon, G. M. and Cuatrecasas, P. (1981b) *Proc. Natl. Acad. Sci. U.S.A.* 78, 6158-6162
- Salamon, Y. (1979) *Advances in Cyclic Nucleotide Research* 10, 35-56
- Sandvig, K. and Olsnes, S. (1981) *J. Biol. Chem.* 256, 9068-9076
- Sattler, J. and Weingadt, H. (1975) *Europ. J. Biochem.* 57, 309-316
- Schlegel, W., Kempner, W. S. and Rodbell, M. (1979) *J. Biol. Chem.* 254, 5168-5176
- Schleifer, L. S., Garrison, J. C., Sternweis, P. C., Northup, J. K. and Gilman, A. G. (1980) *J. Biol. Chem.* 255, 2641-2644
- Schleifer, L. S., Kahn, R. A., Hanski, E., Northup, J. K., Sternweis, P. C. and Gilman, A. G. (1982) *J. Biol. Chem.* 257, 20-23
- Segrest, J. P. and Feldman, R. J. (1974) *J. Molec. Biol.* 87, 853-858
- Sigler, P. G., Druyan, M. E., Kiefer, H. C. and Finkelstein, R. A. (1977) *Science* 197, 1277-1279
- Spicer, E. K., Kavanaugh, W. M., Dallas, W. S., Falkow, S., Konigsberg, W. S. and Schafer, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 50-54
- Stark, G. R. (1977) *Methods Enzymol.* 47, 129-132
- Sternweis, P. G., Northup, J. K., Smigel, D. M. and Gilman, A. G. (1981) *J. Biol. Chem.* 256, 11517-11526
- Streuli, C. H., Patel, B. and Critchley, D. R. (1981) *Exp. Cell. Res.* 136, 247-254

- Studier, F. W. (1973) *J. Molec. Biol.* 79, 237-248
- Svoboda, M., Lambert, M. and Christophe, J. (1981a) *Biochim. Biophys. Acta* 675, 46-61
- Svoboda, M., Furnelle, J. and Christophe, J. (1981b) *FEBS Lett.* 135, 207-211
- Swanstrom, R. and Shank, P. R. (1978) *Anal. Biochem.* 86, 184-192
- Tait, R. M. (1980) Ph.D. thesis, University of Edinburgh
- Tait, R. M. (1981) *Biochemical Society Transactions* 9, 206P
- Tait, R. M. and van Heyningen, S. (1978) *Biochem. J.* 175, 1059-1062
- Tait, R. M., Booth, B. R. and Lambert, P. A. (1980) *Biochem. Biophys. Res. Commun.* 96, 1024-1031
- Takahashi, K. (1968) *J. Biol. Chem.* 243, 6171-6179
- Tanford, C. (1962) *J. Amer. Chem. Soc.* 84, 4240-4247
- Tolan, D. R., Lambert, J. M., Boileau, G., Ganning, T. G., Kenny, J. W., Vassos, A. and Traut, R. R. (1980) *Anal. Biochem.* 103, 101-109
- Tolkovsky, A. M., Braun, S. and Levitzki, A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 213-217
- Tomasi, M. and Montecucco, C. (1981) *J. Biol. Chem.* 256, 11177-11181
- Tosteson, M. T. and Tosteson, D. C. (1978) *Nature (London)* 275, 142-144
- Trepel, J. B., Chuang, D. M. and Neff, N. H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5440-5442
- Trepel, J. B., Chuang, D. M. and Neff, N. H. (1981) *J. Neurochem.* 36, 538-543
- Tsuru, S., Matsuguchi, M., Zinnaka, Y. and Takeya, K. (1982) *J. Gen. Microbiol.* 128, 497-502
- Ueda, K., Hayashi, O., Kawaichi, M., Ogata, N., Ikai, K., Oka, J. and Okayama, H. (1979) in "Modulation of protein function" (ICN-UCLA symposia on molecular and cellular biology, vol. 13) pp47-64, Academic Press, London and New York
- van Heyningen, S. (1974) *Science* 183, 656-657
- van Heyningen, S. (1976a) *J. Infec. Dis.* 133, S5-S13
- van Heyningen, S. (1976b) *Biochem. J.* 157, 785-787
- van Heyningen, S. (1977a) *Biol. Rev. Cambridge Phil. Soc.* 52, 509-549

- van Heyningen, S. (1977b) *Biochem. J.* 168, 457-463
- van Heyningen, S. (1980) in "Enzymology of post-translational modification of proteins" (Freedman, R. B. and Hawkins, H. C. eds.), vol. 1, pp.389-422, Academic Press, London
- van Heyningen, S. (1981) *Nature (London)* 292, 293-294
- van Heyningen, S. (1982a) *Europ. J. Biochem.* 122, 333-337
- van Heyningen, S. (1982b) *Bioscience Reports* 2, 135-146
- van Heyningen, S. and King, C. A. (1975) *Biochem. J.* 146, 269-271
- van Heyningen, S. and Tait, R. M. (1980) in "Hormones and cellular regulation" (Dumont, J. and Nunez, J. eds.) vol. 4, pp293-309, Elsevier - North Holland, Oxford and Amsterdam
- van Heyningen, W. E., Carpenter, C. C. J., Pierce, N. F. and Greenhough, W. B. (1971) *J. Infec. Dis.* 124, 415-418
- Van Ness, B. G., Howard, J. B. and Bodley, J. W. (1978) *J. Biol. Chem.* 253, 8687-8690
- Van Ness, B. G., Howard, J. B. and Bodley, J. W. (1980) *J. Biol. Chem.* 255, 10710-10716
- Varandani, P. T., Raveed, D. and Nafz, M. A. (1978) *Biochim. Biophys. Acta* 538, 343-353
- Vitti, P., De Wolf, M. J. S., Acquaviva, A. M., Epstein, M. and Kohn, L. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 1525-1529
- Walaas, O., Horn, R. S., Lystad, E. and Adler, A. (1981) *FEBS Lett.* 128, 133-136
- Watkins, P. A., Moss, J. and Vaughan, M. (1981) *J. Biol. Chem.* 256, 4895-4899
- Wattiaux-De Coninck, S., Dubois, F. and Wattiaux, R. (1981) *FEBS Lett.* 123, 33-36
- Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406-4412
- Weber, K., Pringle, J. R. and Osborn, M. (1972) *Methods Enzymol.* 26, 3-27
- Welton, A. F., Lad, P. M., Newby, A. C., Yamamura, H., Nicosia, S. and Rodbell, M. (1977) *J. Biol. Chem.* 252, 5947-5950
- Wisnieski, B. J. and Bramhall, J. S. (1979) *Biochem. Biophys. Res. Commun.* 87, 308-313
- Wisnieski, B. J., Shiflett, M. A., Mekalanos, J. and Bramhall, J. S. (1979) *Journal of Supramolecular Structure* 10, 191-197

Wodnar-Filipowicz, A. and Lai, C. Y. (1976) Arch. Biochem. Biophys. 176, 465-471

Wold, F. (1967) Methods Enzymol 11, 617-640

Yamaizumi, M., Makeda, E., Uchida, T. and Okada, Y. (1978) Cell 15, 245-250

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ACTION OF CHOLERA TOXIN

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Cholera toxin, a potent activator of adenylate cyclase in almost all types of eukaryotic cell, is made up of two components: one subunit A (itself two polypeptide chains, A1 (mol. wt 22 000) and A2 (5 000)), and five subunits B (11 500). Subunit B binds to ganglioside GM1 on the outer membrane of susceptible cells, and, in some way not understood, this allows the A1 chain to catalyse a presumably cytoplasmic reaction: the ADP-ribosylation of a GTP-binding protein in the cyclase complex. Toxin in which the subunits had been covalently cross-linked remained active. Proteolytic fragments of the toxin were active, but experiments using ³H-labelled toxin suggested that they were not formed in vivo enough to account for the activity. Toxin bound to nylon strips was not active even with lysed cells. Pigeon erythrocytes whose membranes had been artificially depleted of cholesterol, so increasing their fluidity, showed a much shorter than usual lag phase before cyclase activity increased. These and other observations suggest that the entry of some active part of A1 may be a comparatively non-specific process. The activity of the toxin in rat liver membranes has been studied. No cytoplasmic components are required. Studies with ³²P -NAD⁺ show that only a few proteins are ADP-ribosylated; the molecular weights of the major bands were 43 000 and 49 000. Reconstitution experiments suggested that these were regulatory, not catalytic components. The reaction is not reversible. In a solubilized system, the toxin seems to act as an inhibitor of endogenous ADP-ribosylation.

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THE TRANSMEMBRANE ACTION OF CHOLERA TOXIN IS
MEDIATED BY HYDROPHILIC SUBUNITS.

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The amino-acid compositions suggested that whole cholera toxin and subunit A would be hydrophilic and subunit B would be amphiphilic. The subunits were purified either by sequential elution from an insoluble toxin-ganglioside-cerebroside complex, or by low pH treatment and chromatography on phosphocellulose in 6M urea. On charge shift electrophoresis, whole toxin and subunit A remained anionic and subunit B remained cationic in the presence of both ionic (deoxycholate, CTAB) and non ionic (Triton X-100) detergents, showing that all three proteins are hydrophilic in their native conformation. During their action, subunits A and B interact with polar molecules on different sides of the cell membrane, but how subunit A crosses the membrane is not clear.

The hydrophobicities of cholera toxin, tetanus toxin and their components

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1. Charge-shift electrophoresis showed that cholera toxin and its subunits have no hydrophobic surfaces. 2. Amino-acid composition and sequence data suggested that the proteins have no masked hydrophobic regions. 3. The A subunit of cholera toxin may interact with polar molecules in the membrane to exert its effect inside the cell. 4. The only hydrophobic part of tetanus toxin was the H-chain.

Cholera toxin activates adenylate cyclase in most eukaryotic cells (van Heyningen, 1977; Moss & Vaughan, 1979). The molecule consists of a ring of five B subunits, and one A subunit. The B subunits (M_r about 11500) bind specifically to the oligosaccharide moiety of ganglioside G_{M1} on the outer surface of susceptible cells. The A subunit consists of two peptides, which are joined by a disulphide bond; peptide A2 (M_r about 5000) is in close contact with the B subunits and peptide A1 (M_r about 22000) catalyses the ADP-ribosylation of a regulatory component of the adenylate cyclase complex. As this reaction occurs inside the cell, the A1 peptide (or some part of it) must be transported across the plasma membrane.

Little is known about this transport process. Since only a few molecules of peptide A1 are probably needed inside the cell to activate the cyclase, it need not be very efficient. There is a lag phase between the initial binding of the toxin to the surface of intact cells and the subsequent increase in the cyclase activity. This may be due to the time taken for the toxin to penetrate the membrane, or to interact with membrane proteins, or for the disulphide bond in subunit A to be reduced, or for the multivalency for ganglioside G_{M1} to be satisfied, or for a combination of these factors. Photoaffinity labelling experiments show that the A1 peptide is the only part of the toxin that enters the lipid bilayer (Wisniewski & Bramhall, 1981).

In the present paper, we describe experiments showing that whole cholera toxin and both its subunits have hydrophilic exteriors and so are unlikely to interact easily with side chains of fatty acids in the membrane. Theoretical calculations

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based on the amino-acid composition and sequence of the proteins support this conclusion.

Much less is known about the action of tetanus toxin, which interacts with neuronal cells by an unknown mechanism (van Heyningen, 1980). It is made up of two chains linked by a disulphide bond. The H-chain (M_r about 100000) binds to a ganglioside on the cell surface, but the L-chain (M_r 50000) does not bind. Our experiments show that tetanus toxin is also hydrophilic, although the H-chain has some hydrophobicity.

Experimental

Materials

Cholera toxin was purified from culture filtrates of *Vibrio cholerae* kindly grown for us at the Centre of Applied Microbiology and Research, Porton, Wilts., U.K. The subunits were purified by chromatography on Sephadex G-75 (Pharmacia, Hounslow, Middx., U.K.) in urea at low pH (Moss *et al.*, 1976). Tetanus toxin was purified (van Heyningen, 1976) from material kindly given to us by Dr. R. O. Thomson of the Wellcome Research Laboratories, Beckenham, Kent, U.K.

Charge-shift electrophoresis

Hydrophobicity was measured experimentally by the method of Helenius & Simons (1977). The binding of the non-ionic detergent Triton X-100 (Koch-Light, Colnbrook, Bucks., U.K.) to the protein is observed by determining the charge on the protein in detergent mixtures containing Triton and another anionic or cationic detergent. Proteins with hydrophobic areas on their surface bind Triton, and so migrate in different directions in each detergent mixture when electrophoresed in 1% agarose.

Assay of cholera-toxin activity

Cholera toxin and its subunits were pre-incubated with the detergents and then assayed for the ability to activate adenylate cyclase in rat liver membranes (Tait *et al.*, 1980) and for binding to insoluble ganglioside/cerebroside mixtures (van Heyningen, 1974).

Results

Predicted hydrophobicities

There are several different methods for estimating the hydrophobicity of a protein from its amino-acid

composition (Bigelow, 1967; Barrantes, 1975), or sequence (Segrest & Feldman, 1974). The results are expressed in terms of different parameters and compared with values calculated for proteins of known hydrophobicities (Table 1). Such predictions could indicate the existence of masked hydrophobic areas that would not be detected by charge-shift electrophoresis. All the methods suggested that cholera toxin and both its subunits are probably hydrophilic, although perhaps with a little amphiphilic character especially in peptide A2. There were several hydrophobic sequences in the toxin and in the very similar A subunit of *Escherichia coli*

Table 1. *Hydrophobicities of cholera toxin and its subunits predicted from their amino-acid compositions*
The ranges of the hydrophobicity estimates for standard proteins were calculated by Barrantes (1975) and Raftery *et al.* (1975). The range quoted represents the mean \pm s.d., and *n* indicates the number of proteins studied. Data on the structure of cholera toxin are reviewed by Lai (1980); 1 cal \approx 4.18 J.

Protein	Estimates of parameters of hydrophobicity		
	\bar{H}_o (kcal/mol) (Bigelow, 1967)	R_3 (Barrantes, 1975)	z (Barrantes, 1975)
Non-membrane proteins (<i>n</i> = 205)	0.90–1.09	0.84–1.68	–0.10 to 0.33
External membrane proteins (<i>n</i> = 24)	0.91–1.06	1.02–1.72	–0.04 to 0.28
Internal membrane proteins (<i>n</i> = 24)	1.10–1.29	0.41–0.77	0.41–0.63
Toxin			
A1 peptide	0.94–1.07	1.29	0.12–0.20
A2 peptide	0.78–0.97	1.72	–0.13 to –0.01
A subunit	0.90–1.04	1.40	0.05–0.14
B subunit	1.05	1.34	0.16–0.17
A2–(B) ₅ complex	1.02–1.04	1.39	0.13–0.15
Whole toxin [i.e. A1–A2–(B) ₅]	1.00–1.05	1.36	0.13–0.16

Table 2. *Migration of proteins on charge-shift electrophoresis*

Abbreviation: CTAB, cetyltrimethylammonium bromide. + indicates anodal migration; – indicates cathodal migration; 0 indicates no migration; * indicates that under these conditions, diphtheria toxin split into two components (see Boquet, 1979).

Detergent mixture	Direction of migration				Were hydrophobic areas detected on the protein surface?
	No detergent	Triton alone	Triton + cholate	Triton + CTAB	
Standard proteins					
Bovine serum albumin	+	+	+	+	No
Ovalbumin	+	+	+	+	No
Diphtheria toxin	+	+	+	+, –*	No
Immunoglobulin G	0	0	0	0	No
Human apoprotein B from low-density lipoprotein	+	0	+	–	Yes
Human apoprotein A1 from high-density lipoprotein	+	0	+	–	Yes
Cytochrome <i>b</i> ₅₆₁	+	0	+	Not determined	Yes
Toxins					
Whole cholera toxin	+	+	+	+	No
Subunit A	+	+	+	+	No
Subunit B	–	–	–	–	No
Whole tetanus toxin	+	+	+	+	No
H-chain	+	0	+	–	Yes
L-chain	+	+	+	+	No

heat-labile toxin (Spicer *et al.*, 1981). But the hydrophobicity indices were comparable with those found for the interiors of proteins rather than for hydrophobic proteins (Segrest & Feldman, 1974).

Charge-shift electrophoresis of cholera toxin

The charge-shift system was tested with several proteins of known hydrophobicity. Cholera toxin and both of its subunits migrated as proteins with no hydrophobic areas on their surface (Table 2).

Activity of cholera toxin

After pretreatment with the detergents used in the charge-shift experiments, cholera toxin and subunit A both retained the ability to activate the adenylate cyclase of rat liver membranes. They had similar specific activity to the native proteins. Whole toxin and subunit B both bound to ganglioside G_{M1} , insolubilized by complexing it to cerebroside, but not to cerebroside alone.

Tetanus toxin

In similar charge-shift experiments, tetanus toxin migrated as a hydrophilic protein (Table 2). Investigation of the component chains was complicated by the difficulty of preparing them pure in the absence of sodium dodecyl sulphate. Also the H-chain requires urea to keep it in solution. However, on charge-shift electrophoresis in the presence of 2-mercaptoethanol, the toxin [labelled with ^{125}I] (Greenwood *et al.*, 1963) and in the presence or absence of 4M-urea split into two components, which were identified by elution and analytical electrophoresis. The L-chain migrated with whole toxin as a hydrophilic protein, but the H-chain apparently has some hydrophobic areas on its surface.

Discussion

These experiments show that the surfaces of cholera toxin and its subunits are all hydrophilic, and the theoretical calculations suggest that there are no masked hydrophobic regions in the A1 peptide or the B subunit. If, as the calculations suggest, the A2 peptide (which is very difficult to work with experimentally after purification) is amphiphilic, its hydrophobic areas must be masked in the intact A subunit. The A2 peptide could act as a hydrophobic leader sequence for the entry of peptide A1 into the plasma membrane. This would correlate with the observation that a fluorescent derivative of A subunit showed a conformational change when toxin bound ganglioside G_{M1} (S. van Heyningen, unpublished work). But the A2 peptide is not strikingly hydrophobic and was not detected in the lipid bilayer by photoaffinity labelling (Wisniewski & Bramhall, 1981).

These results do not support suggestions that the A1 chain partitions directly into the cell membrane. But, as only very small amounts are needed inside the cell, such passage need not be a very likely event. Since A1 is hydrophilic, it could enter the membrane (where it was detected by photoaffinity labelling), after interaction with some integral membrane protein. There is evidence that the action of the toxin requires certain membrane proteins (Hagmann & Fishman, 1981). To exert its transmembrane effect, the A subunit may interact with the substrate of the toxin, other components of adenylate cyclase or protein disulphide reductase (Moss *et al.*, 1980). Alternatively the toxin may be taken up by endocytosis, but the A1 peptide would still have to cross the lysosomal membrane to activate adenylate cyclase. There is some indirect evidence that the protein does not cross this membrane, but rather reaches its target as a result of lysosomal processing (Houslay & Elliott, 1981). This work needs further investigation. The hydrophilic B subunit was not detected in the membrane by photoaffinity labelling.

The observed hydrophobicity of the H-chain of tetanus toxin suggests it could dissolve in the membrane. This correlates with evidence (from limited trypsinolysis in urea) for some conformational change when the toxin (which has a hydrolytic surface) binds ganglioside (P. Britton & S. van Heyningen, unpublished work).

The transmembrane action of cholera toxin probably involves a localized destabilization of the membrane due to multivalent binding of ganglioside G_{M1} . The B subunits would not enter the membrane, but a very high local concentration of A subunits would be induced. A very small proportion of the A1 peptides could then interact with polar molecules in the membrane to exert their effect on the inside of the cell.

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References

- Barrantes, F. J. (1975) *Biochem. Biophys. Res. Commun.* **62**, 407–414
- Bigelow, C. C. (1967) *J. Theor. Biol.* **161**, 187–211
- Boquet, P. (1979) *Eur. J. Biochem.* **100**, 483–489
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Hagmann, J. & Fishman, P. H. (1981) *Biochem. Biophys. Res. Commun.* **98**, 677–684
- Helenius, A. & Simons, K. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 529–532
- Houslay, M. D. & Elliott, K. R. F. (1981) *FEBS Lett.* **128**, 289–292

- Lai, C. Y. (1980) *Crit. Rev. Biochem.* **9**, 171-206
- Moss, J. & Vaughan, M. (1979) *Annu. Rev. Biochem.* **48**, 581-660
- Moss, J., Manganiello, V. C. & Vaughan, M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4424-4427
- Moss, J., Stanley, S. J., Morin, J. E. & Dixon, J. E. (1980) *J. Biol. Chem.* **255**, 11085-11087
- Raftery, M. A., Vandlen, R. L., Reid, K. L. & Lee, T. (1975) *Cold Spring Harbor Symp. Quant. Biol.* **40**, 193-202
- Segrest, J. P. & Feldman, R. J. (1974) *J. Mol. Biol.* **87**, 853-858
- Spicer, E. K., Kavanaugh, W. H., Dallas, W. S., Falkow, S., Konigsberg, W. H. & Schafer, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 50-54
- Tait, R. M., Booth, B. R. & Lambert, P. A. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1024-1031
- van Heyningen, S. (1974) *Science* **183**, 656-657
- van Heyningen, S. (1976) *FEBS Lett.* **68**, 5-7
- van Heyningen, S. (1977) *Biol. Rev. Cambridge Philos. Soc.* **52**, 504-549
- van Heyningen, S. (1980) *Pharmac. Ther.* **11**, 141-157
- Wisniewski, B. J. & Bramhall, J. S. (1981) *Nature (London)* **289**, 319-321

THE SITES ON THE REGULATORY COMPONENT OF ADENYLATE CYCLASE WHICH ARE
ADP-RIBOSYLATED BY CHOLERA TOXIN

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In rat liver membranes cholera toxin ADP-ribosylated two polypeptides (M_r 42000 and 47000) in the regulatory component of adenylate cyclase. L-arginine methyl ester specifically inhibited both the activation of adenylate cyclase and ADP-ribosylation by cholera toxin, suggesting that cholera toxin modified arginine, or arginine-like, residues. A hydrolysis-resistant analogue of GTP (β , γ -imidoguanosine 5'-triphosphate, p(NH)ppG) bound to the regulatory protein in an essentially irreversible manner. Pretreatment with the analogue failed to inhibit the labelling of polypeptides by cholera toxin showing that the sites for ADP-ribosylation were different from those at which guanyl nucleotides were bound.

INTRODUCTION

Cholera toxin activates adenylate cyclase by inhibiting a GTPase which is a regulatory component of the enzyme (1). In rabbit liver, cholera toxin catalysed the transfer of ADP-ribose from NAD^+ to two polypeptides (M_r 45000 and 52000) in this protein (2). After stimulation by a hydrolysis-resistant analogue of GTP (p(NH)ppG), adenylate cyclase could not be further stimulated by cholera toxin (3). It is therefore possible that cholera toxin could modify groups which are involved in the binding site for guanyl nucleotides on the regulatory protein.

Arginine and other small molecules containing a guanidinium group stimulate the ability of preparations of cholera toxin to catalyse the release of nicotinamide from NAD^+ (4). N.m.r. studies showed that cholera toxin catalysed the ADP-ribosylation of arginine (5). However, there is little evidence to suggest that cholera toxin

Abbreviations: p(NH)ppG, β , γ -imidoguanosine 5'-triphosphate;
SDS, sodium dodecyl sulphate; PAGE, polyacrylamide-gel electrophoresis.

interacts with arginine residues in proteins (6, 7). The abilities of compounds that are related to arginine to inhibit the action of cholera toxin was therefore investigated.

EXPERIMENTAL

Materials. p(NH)ppG and molecular-weight markers were obtained from Sigma London Chemical Co. Ltd, Poole, U.K.. Radiochemicals were supplied by Amersham International, Amersham, U.K.. Cholera toxin was purified (by a method modified from Mekalanos *et al.*, 8) from culture filtrates of *Vibrio cholerae* kindly grown for us at the Centre for Applied Microbiology and Research, Porton, U.K..

Rat liver membranes and incubation with cholera toxin. A crude membrane preparation from the livers of female rats was made by the method of Tait *et al.* (9). Fresh preparations were incubated with cholera toxin then washed (9). Samples were assayed in duplicate for adenylate cyclase activity (10). Duplicate assays agreed to within $\pm 5\%$. One sample in each set of experiments was incubated with 20 mM sodium fluoride to test for functional adenylate cyclase and regulatory protein. In ADP-ribosylation experiments [adenylate- ^{32}P] NAD $^{+}$ was used at about 40 Ci/mmol and 10 μM . Protein concentrations were measured by the method of Bradford (11) using bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis (PAGE) and autoradiography. PAGE in the presence of 0.1% (w/v) sodium dodecylsulphate (SDS) was performed by the method of Laemmli (12). The separating gel was 8% (w/v) in acrylamide. The molecular-weight markers used were phosphorylase b (94000), bovine serum albumin (66500) pyruvate kinase (57000), ovalbumin (43000), yeast alcohol dehydrogenase (37000) and myoglobin (17200). The gels were dried under vacuum with heating, then exposed to Agfa Curix RPL X-ray film between Dupont Cronex Lightning Plus intensifying screens (Dupont (U.K.) Ltd, Stevenage, U.K.) at -70°C for 2 days. Autoradiographs were scanned for extinction at 600 nm, and the amount of radioactivity estimated from the area under the peaks. This enabled estimation of label incorporated and M_r of radioactive polypeptides both to within $\pm 10\%$.

RESULTS AND DISCUSSION

The results of our experiments investigating the mechanism of action of cholera toxin on rat liver were consistent with the model which was described for other tissues in the introduction. Cholera toxin catalysed the specific ADP-ribosylation of two polypeptides (M_r 42000 and 47000) (fig. 1) in the regulatory component of adenylate cyclase. Different workers have detected different substrates of cholera toxin. A single substrate (M_r 42500) of cholera toxin was found (13) in a different preparation of rat liver membranes with different incubation conditions. Receptors of M_r 42000 and 47000 were detected in human fibroblasts (14).

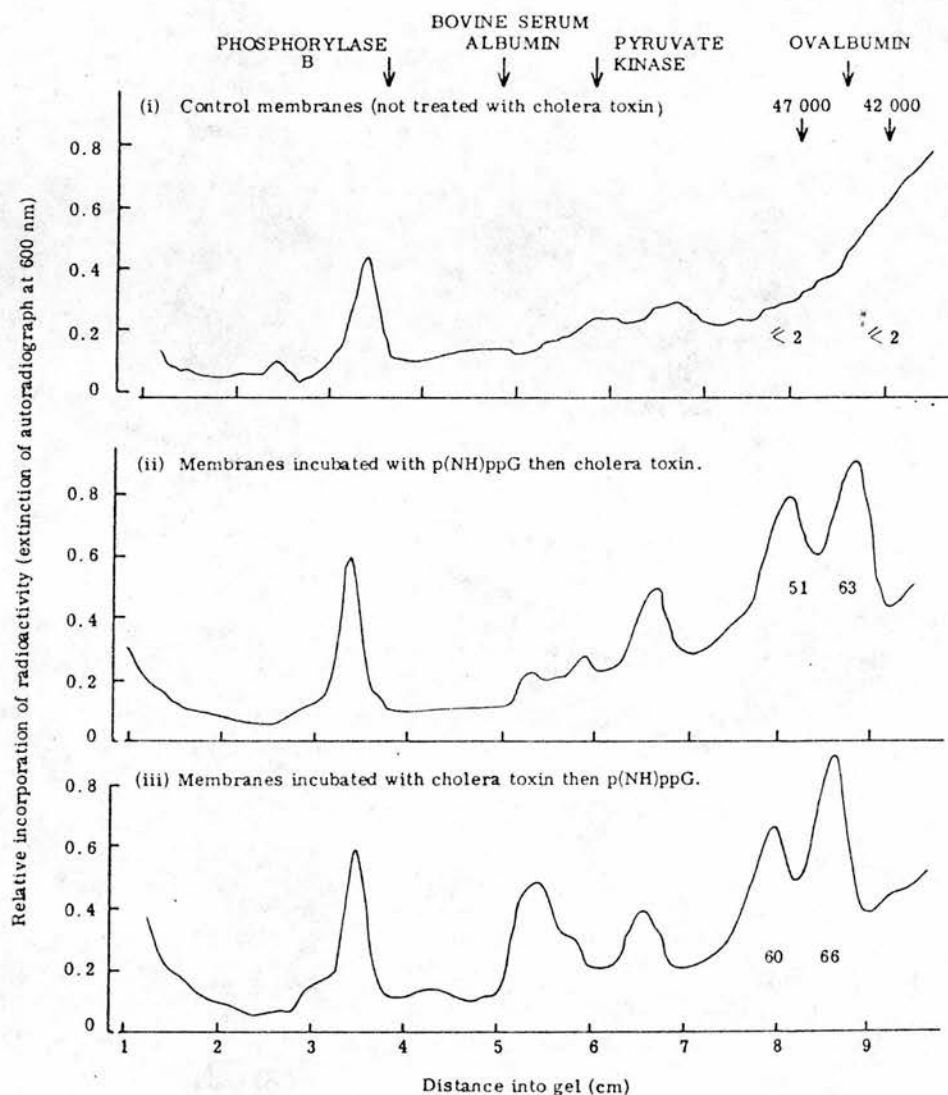


Fig. 1

The labelling of membrane polypeptides by cholera toxin and [adenylate- ^{32}P] NAD $^{+}$

After incubation with [^{32}P] NAD $^{+}$, membrane proteins were analysed by SDS-PAGE. Radioactive polypeptides were identified on autoradiographs which were scanned for extinction to measure the amount of labelling. The figures under the peaks represent the incorporation of ADP-ribose (fmol/mg protein).

Because of its ionic strength, arginine methyl ester at low concentrations increased both basal and toxin-stimulated activities of adenylate cyclase. However, high concentrations blocked the action of cholera toxin (fig. 2). The stimulation of adenylate cyclase by fluoride ions was not inhibited by arginine methyl ester

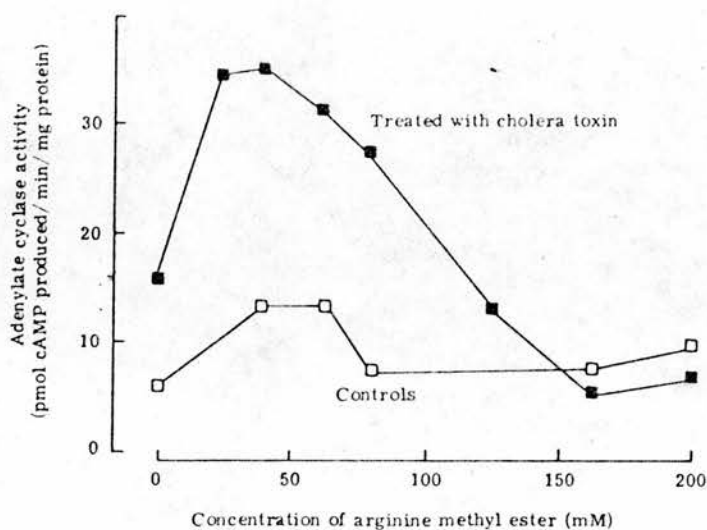


Fig. 2

The effect of L-arginine methyl ester on the activation of adenylate cyclase by cholera toxin

(table 1). Several other compounds that are structurally related to arginine methyl ester were also tested for the ability to inhibit cholera toxin. Since they could affect the stability of adenylate cyclase, the ratio of stimulation of adenylate cyclase activity by cholera toxin to the stimulation of activity by fluoride was used as a measure of specific blocking of the toxin. At concentrations where arginine methyl ester completely blocked the effect of cholera toxin, only lysine methyl ester caused partial inhibition (table 1).

High concentrations of arginine methyl ester or lysine methyl ester blocked the toxin-specific, but not the endogenous, labelling of membrane proteins using [adenylate- ^{32}P] NAD^+ . This was not an effect of ionic-strength as it could not be mimicked by NaCl or citrulline. The effects of different compounds on the activation of adenylate cyclase by cholera toxin paralleled the effects on ADP-ribosylation (table 1). Slight effects of the toxin were more easily detected in the adenylate cyclase assay than in the ADP-ribosyl transferase assay as the former system produces a catalytically active species. These results are similar to the effects of the same compounds on the ability of

Table 1 Involvement of arginine residues in the action of cholera toxin
 Membranes were incubated with [adenylate - 32 P] NAD $^{+}$ then proteins were analysed by SDS-PAGE. Radioactive polypeptides were identified on autoradiographs which were scanned for extinction to measure the amount of labelling. Alternatively membranes were incubated with unlabelled NAD $^{+}$, washed then adenylate cyclase activity was assayed. ND = not determined.

Additions to incubation with toxin	Labelling of polypeptides (fmol ADP-ribose/mg protein)		Adenylate cyclase activity (pmol cAMP produced/min/mg protein)		
	M _r 47 000	M _r 42 000	basal (a)	stimulated by toxin (b)	stimulated by fluoride (c)
Experiment (i)					
none	20	33	11.4	22.4	149.2
250 mM NaCl	27	36	10.0	19.0	139.0
150 mM L-arginine methyl ester	≤2	≤2	14.9	14.4	149.1
150 mM guanidine	ND	ND	6.0	5.1	42.7
150 mM L-lysine methyl ester	≤2	≤2	9.0	13.8	127.8
150 mM L-citrulline	26	44	11.4	20.3	135.0
Experiment (ii)					
none	20	33	15.8	39.4	134.0
150 mM L-histidine	ND	ND	15.6	37.0	149.6
150 mM L-arginine methyl ester	≤2	≤2	15.8	15.6	131.0
					≤0
					0.20
					0.16
					≤0
					0.08
					0.07
					≤0
					≤0
					0.04
					0.07

cholera toxin preparations to catalyse the release of nicotinamide from NAD^+ (5).

Our results suggest that cholera toxin may be interacting with arginine or some arginine-like residue on the protein which regulates adenylate cyclase. The specificity of labelling of proteins may be because cholera toxin recognises an unusual or modified amino-acid residue in its substrates, like the modified histidine residue which is ADP-ribosylated by diphtheria toxin (15). Cholera toxin may recognise a specific sequence of amino-acids or three-dimensional structure on its substrates (16).

In rat hepatocytes, arginine decreased ADP-ribosylation of a polypeptide of M_r 55000 by cholera toxin (6). Gill (7) found that arginine inhibited the activation of adenylate cyclase in pigeon erythrocytes by cholera toxin, but it had no detectable effect on the incorporation of ^{32}P from NAD^+ into proteins. Differences between our results and those of other workers may be because methyl esters are better models than free amino-acids for residues in proteins, or because in our work inhibitors were used at much higher concentrations than in other studies, or because of differences between the experimental systems.

To test if cholera toxin ADP-ribosylated the groups which are involved in the binding site for guanyl nucleotides on the regulatory protein, membranes were incubated with 1 mM p(NH)ppG for 20 min, washed, and then treated with cholera toxin and [adenylate- ^{32}P] NAD^+ . In control experiments the sequence of the two incubations was reversed. Pretreatment of membranes with p(NH)ppG failed to block ADP-ribosylation by cholera toxin (fig. 1). This observation is consistent with the observed requirement for GTP in the action of cholera toxin (13). But, as exchange of guanyl nucleotides occurs at the regulatory site, GTP could dissociate to allow access for cholera toxin. After preincubation with [8- ^3H] p(NH)ppG, washing and solubilization, no

dissociation of ^3H was detectable by gel-permeation chromatography. Together with other experiments, this showed that p(NH)ppG probably remained bound to essentially all of its binding sites on the regulatory protein.

p(NH)ppG failed to block ADP-ribosylation by cholera toxin, showing that the site of labelling is not the binding site for guanyl nucleotides on the regulatory protein. This result correlated with the observation that cholera toxin modifies the function of initiation factor 2 (eIF-2) in protein synthesis by ADP-ribosylating the β subunit (17). The α subunit of eIF-2 binds GTP. These facts suggest a general mechanism for the modification of regulatory GTPases by cholera toxin at sites where guanyl nucleotides are not bound.

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REFERENCES

- (1) Cassell, D. and Selinger, Z. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 3307-3311.
- (2) Northup, J.K., Sternweis, P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M. and Gilman, A.G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 6516-6520.
- (3) Flores, J. and Sharp, G.W.G. (1975) *J. Clin. Invest.* 56, 1345-1349.
- (4) Moss, J. and Vaughan, M. (1977) *J. Biol. Chem.* 252, 2455-2457.
- (5) Oppenheimer, N.J. (1978) *J. Biol. Chem.* 253, 4907-4910.
- (6) Beckner, S.K. and Blecher, M. (1981) *Biochem. Biophys. Acta* 673, 477-486.
- (7) Gill, D.M. (1979) *Journal of Supramolecular Structure* 10, 151-163.
- (8) Mekalanos, J.J., Collier, R.J. and Romig, W.R. (1978) *Infect. Immun.* 20, 552-558.
- (9) Tait, R.M., Booth, B.R. and Lambert, P.A. (1980) *Biochem. Biophys. Res. Commun.* 96, 1024-1031.
- (10) Salamon, Y. (1979) *Advances in Cyclic Nucleotide Research* 10, 35-56.
- (11) Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254.
- (12) Laemmli, U.K. (1970) *Nature* 227, 680-685.
- (13) Doberska, C.A., MacPherson, A.J.S. and Martin, B.R. (1980) *Biochem. J.* 186, 749-754.
- (14) Watkins, P.A., Moss, J. and Vaughan, M. (1981) *J. Biol. Chem.* 256, 4895-4899.
- (15) van Ness, B.J., Howard, J.B. and Bodley, J.W. (1980) *J. Biol. Chem.* 255, 10717-20.
- (16) Hudson, T.H. and Johnson, G.L. (1980) *J. Biol. Chem.* 255, 7480-7486.
- (17) Cooper, D.M.F., Jagus, R., Somers, R.L.S. and Rodbell, M. (1981) *Biochem. Biophys. Res. Commun.* 101, 1179-1185.